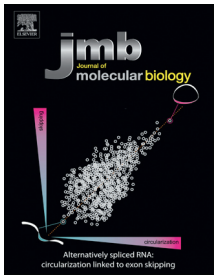

Tools and Principles for Microbial Gene Circuit Engineering

Robert W. Bradley [1,2], Martin Buck [1] and Baojun Wang [2,3]

*1 - **Department of Life Sciences,** Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, United Kingdom*
*2 - **School of Biological Sciences,** University of Edinburgh, Edinburgh EH9 3FF, United Kingdom*
*3 - **Centre for Synthetic and Systems Biology,** University of Edinburgh, Edinburgh EH9 3FF, United Kingdom*

***Correspondence to Baojun Wang:** School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FF, United Kingdom. Baojun.Wang@ed.ac.uk*
http://dx.doi.org/10.1016/j.jmb.2015.10.004
Edited by T. K. Lu

Abstract

Synthetic biologists aim to construct novel genetic circuits with useful applications through rational design and forward engineering. Given the complexity of signal processing that occurs in natural biological systems, engineered microbes have the potential to perform a wide range of desirable tasks that require sophisticated computation and control. Realising this goal will require accurate predictive design of complex synthetic gene circuits and accompanying large sets of quality modular and orthogonal genetic parts. Here we present a current overview of the versatile components and tools available for engineering gene circuits in microbes, including recently developed RNA-based tools that possess large dynamic ranges and can be easily programmed. We introduce design principles that enable robust and scalable circuit performance such as insulating a gene circuit against unwanted interactions with its context, and we describe efficient strategies for rapidly identifying and correcting causes of failure and fine-tuning circuit characteristics.

Introduction

Microbes are capable of sensing a wide variety of stimuli, processing information efficiently, and producing a range of chemical and physical responses. Genetic engineers have repurposed these impressive capabilities to interrogate natural biological signalling pathways [1] and have reprogrammed cells to produce desirable compounds [2], to sense and report on the presence of toxic metals [3] or cancer [4], or to seek and destroy pathogenic bacteria [5,6]. These state-of-the-art examples use relatively simple signal processing networks compared to complex native systems, hinting at the great potential for designer organisms but also highlighting how difficult it currently is to effectively and routinely program synthetic gene circuits [7,8].

Synthetic biology has often adopted an electrical engineering framework for the design of novel gene networks: basic functional units are termed parts [9,10] and can be connected to build circuits, input–output responses are described as having analogue or digital characteristics [11,12], and signal processing is often implemented using Boolean logic functions [13]. This conceptual framework is effective up to a point, but predictions of how gene circuits will behave often fail when they are implemented in living cells because of the many undefined, complex and dynamic interactions that can occur between the circuit and its context [14,15]. Functional circuits can still be produced, using iterative cycles of testing and refinement to correct failures, and strategies for insulating parts from confounding contextual effects are being developed [16,17], but the inability to accurately model circuit behaviour remains a major barrier to the construction of large gene circuits.

Another limiting factor for the construction of complex synthetic gene circuits has been a lack of large sets of appropriately characterised parts, though this has changed in recent years [18–22]. Desirable properties of part families include specificity and orthogonality (strong interactions with

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

cognate parts, without cross-talk), compatibility (can be used in the same circuit), and composability (possessing transfer functions that overlap with other parts) [7]. Parts whose function can be designed *de novo* are particularly amenable to the creation of large orthogonal sets [18–20].

In this review, we describe the current tools and principles available for microbial gene circuit engineering (Table 1), starting with regulators of transcription and translation, two major control points that determine the levels of gene circuit components, and their associated final outputs in a cell. Large sets of orthogonal regulatory parts have been generated [18–22], though recently developed RNA-based tools show particular promise as effective, reusable components for gene circuit engineering. The specificity of RNA-based tools is defined by base-pairing, making design conceptually simple and amenable to computational modelling. Both the CRISPR-dCas9 [19] and the toehold switch [20] part families have dynamic ranges of over two orders of magnitude and have few constraints on their programmability. Tools for post-transcriptional and post-translational signal processing are also discussed, including split inteins for protein splicing [23], the use of scaffolds for coordination of pathways [24], and memory elements [12,25]. Since gene circuits often do not function as expected when they are assembled, we discuss how context effects impact on circuit function [14,15] and how circuits can be insulated against these effects [16,17]. Finally, we look at practical strategies for debugging and tuning circuits to meet design specifications [8,26].

Tools for Engineering RNA Levels

Transcription is arguably the most important control point for a gene circuit. It provides comprehensive regulation over the levels of each component since not all RNAs go on to be translated. The tools available for RNA-based signal processing are increasing in number and capability [18–20,27]. The vectoral nature of transcription gives rise to polar effects, some of which can be useful (e.g., coordination of gene expression as a polycistronic RNA), whilst others are undesirable (read-though of terminators [28]). Control elements are more easily composed at the DNA level [29], avoiding problems that may be caused by secondary-structure formation in mRNA [16,30]. Regulation of transcription affords an efficient use of cellular resources compared to the downstream regulation of translation, as energy and resources are not wasted on RNA synthesis. Here we review and discuss the versatile components, tools, and approaches available to achieve transcription-based gene circuit design in microbial cells. Most transcriptional regulation components control the initiation of RNA synthesis, by modulating RNA polymerase (RNAP) binding affinity with the promoter DNA [31,32], stabilising initiation complex formation [33], or controlling access to a promoter [19,21].

RNAP binding

The nucleotides in a promoter sequence that interact with the RNAP holoenzyme are fundamental determinants of the transcription rate from that site. Sequence-dependent binding occurs between the σ^{70}-type factors and the −10 and −35 (core) regions to specify the initiation site, whilst the C-terminal domains of the α subunits preferentially interact with short tracts of repeated A or T nucleotides in the 30 base pairs upstream of the −35 region (the UP element), influencing promoter strength [34] (Fig. 1a). Various studies have explored how changes to and between the −10 and −35 regions influence σ^{70}-type promoter strength [35,36], with thermodynamic modelling of the binding energies proving one useful predictor for promoter strength [31]. An examination of the contribution of the UP element to σ^{E} promoter strength revealed the importance of RNAP concentration for models of the full-length promoter: the influence of the UP element decreased with increasing RNAP concentration for promoters with a strong core sequence and *vice versa* [37]. The sequence downstream of the transcription start site can also influence transcription rates; potentially interfering effects can be avoided using a standardised 5′ untranslated region (UTR) and a constant initiating nucleotide [17,38]. The libraries of promoter sequences created for these studies and others [17,39,40] span a large range of strengths, making them a useful resource for biological engineers until fully predictive models of promoter activity are built.

Protein transcriptional regulators

Activators

Classical activators used in gene circuit design, such as LuxR [41], work by stabilising RNAP binding to the promoter. Taking a different approach, Rhodius and colleagues used part mining of extracytoplasmic function σ factors (ECFs) to build the largest library of orthogonal protein transcriptional activators available for use in *Escherichia coli*, finding 20 ECFs and cognate promoters that show low cross-reactivity [32]. These proteins function by determining the specificity of RNAP for a given promoter sequence, interacting at the −35 and −10 regions. The authors take advantage of this property to produce synthetic chimeric ECFs, a strategy that they estimate could produce a total set of approximately 160 orthogonal ECF/promoter pairs. ECF

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

function can also be inhibited through their sequestration by an anti-σ factor; thus, anti-σ factors can be used to lower the OFF state and tune the activation of promoters (alter the cooperativity) by their cognate ECF. Effective anti-σ repressors of all 20 orthogonal ECFs were identified, though some cross-reactivity to non-cognate ECFs was observed. Rhodius *et al.* characterise the dynamic range, response function, and toxicity of the parts, making this a rich resource for biological engineers.

Other useful transcriptional activators for use with the native *E. coli* RNAP include three chaperone-activator pairs (which can be used for AND logic) [42], phage transcriptional activators [43], and the HrpRS bacterial enhancer binding proteins [44] (which can be used in combination with the HrpS-inhibiting HrpV protein [45]). HrpRS activates transcription from the σ^{54}-dependent P_{hrpL} promoter in an ATP-dependent manner [46,47]. Hence, contrasting to σ^{70}-dependent transcription, there is no spontaneous open complex formation and P_{hrpL} is not recognised by the σ^{70}-RNAP holoenzyme, which renders the OFF state of the P_{hrpL} promoter close to zero, facilitating a digital-like response upon activation [48].

T7 RNAP

The native *E. coli* RNAP is not necessarily required for transcription: the phage T7 RNAP is a single-subunit enzyme capable of high transcription rates, which recognises promoter sequences that are orthogonal to those used by the native *E. coli* RNAP. Promoter recognition is determined by a "specificity loop" in the enzyme, which a number of groups have modified to produce variants that exhibit differing degrees of selectivity to a given promoter sequence: highly orthogonal sets [49,50] allow for modular control, whilst cross-talk between other combinations of polymerases and promoters could also be useful for differential control of multiple genes [51,52]. The T7 RNAP coding sequence can be split into parts that interact—either spontaneously [51] or more stably via split-intein-mediated *trans*-splicing [23]—to form a functional enzyme. T7 RNAP that is split into two enables basic AND logic [23,51] or analogue addition functions to be incorporated into a gene circuit, though the enzyme can be split into up to four sections [52]. Segall-Shapiro and colleagues demonstrated the use of tripartite T7 RNAP as a "resource allocator", where the expression level of an N-terminal "core" enzyme fragment determines the total level of transcriptional resources available, thereby setting an upper bound on the metabolic load imposed on the cell by actively transcribing synthetic gene circuits [52]. Differential expression of C-terminal "σ" domain fragments that contain orthogonal specificity loops then controls the distribution of the transcriptional resources across an array of target promoters. Positive post-transcriptional regulation of the total T7 RNAP transcriptional resource level is achieved by splitting the core fragment further into "α" and "β" subunits; expression of the α subunit can then be titrated against the constitutively expressed β subunit to control the amount of the holocore fragment. Conversely, sequestration of the core fragment using an inactive version of the σ domain enables negative regulation of total transcription.

Repressors

Transcription-repressing proteins most commonly act by blocking RNAP access to the promoter (Fig. 1a). A library of homologues of the DNA binding protein TetR and their cognate operator sequences was built by Stanton *et al.*, and it contains 16 orthogonal variants sourced from sequence databases [21]. The response function and toxicity of the repressors was characterised, providing useful data for gene circuit design. The authors note that, theoretically, a set of 130 orthogonal variants for this repressor class exists [53]. A smaller library of LacI variants has also been produced [54], which can be used for NAND logic at a promoter due to the cooperative nature of LacI-induced DNA looping.

The DNA binding specificity of certain families of proteins can be predicted and programmed [55,56]: the DNA binding domain of transcription activator-like effectors (TALEs) comprises multiple 34-amino-acid repeats that each have two residues that interact with the DNA major groove. Since the amino acid-nucleotide recognition code is known, repeats can be assembled to create a protein with predictable DNA sequence-specific binding. TALE repressors are more easily programmed compared to zinc-finger DNA binding proteins [57,58], can be used for multiplexed gene control [59], and have been shown to provide over 100-fold repression of gene expression in *E. coli* [60], but to date, they are a poorly represented class of components in *E. coli* gene circuits. This may be due to the relative difficulty in assembling the coding sequence of the binding repeats—though high-throughput methods have been developed for TALE synthesis [61–63]—or possibly due to the emergence of CRISPR-Cas9 technology.

Inducible control of transcription

Small-molecule control: Protein regulators

Transcriptional regulators that are controlled by small-molecule inducers are indispensable to designers of synthetic gene circuits, allowing simple and often cheap external control over mRNA production rates and facilitating cell-to-cell communication in the case of quorum-sensing molecules [64]. A few "classical" regulators (usually proteins) that sense inputs ranging from metabolites to metal

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

Table 1. Tools for microbial gene circuit engineering.

Tool family	Action	References	Notes
Control of RNA levels			
Promoters (RNAP binding)	Control of transcription initiation rate	[31,34–39]	Activity can be reasonably well predicted with thermodynamic models but is sensitive to changes in adjacent sequences
Transcriptional activators			
Extracytoplasmic function σ factors	Recruitment of RNAP to specific promoter sequences	[32]	20 orthogonal ECF/promoter pairs characterised, all with cognate anti-σ factors
HrpRS bacterial enhancer binding proteins; chaperone activators; phage activators	Recruitment of RNAP to specific promoter sequences	[44,45,139,42,43]	HrpRS activate the σ^{54}-dependent P_{hrpL}; ATP dependency gives a low OFF state for a digital-like response
T7 RNAP	Single-subunit phage RNAP recognises orthogonal promoters	[23,49–52]	Variants that recognise orthogonal promoter sequences exist; protein can be split to increase functionality
Transcriptional repressors			
TetR homologues; LacI variants	DNA binding proteins (block promoter binding by RNAP)	[21,54]	16 orthogonal TetR variants characterised with up to 200-fold repressive activity
TALE repressors	DNA binding proteins with programmable sequence specificity	[59–63]	Construction of TALE proteins is relatively time consuming
Small-molecule inducible control of transcription			
Riboswitches	*Cis*-elements that control transcription termination	[70–74]	Only a few small molecules can be sensed
UAA control of transcription	UAA availability controls ribosome stalling in a leader peptide	[78]	Positive regulation is based on *tna* operon control; negative regulation uses *trp* operon attenuator
Optogenetics	Light-sensing two-component systems	[79–82]	Can specifically detect red, green, or blue wavelength
RNA control of transcription			
pT181-based transcription repression	A taRNA induces formation of a transcription terminator	[85,86]	*Cis*-elements can be concatenated
STARs	A taRNA disrupts formation of a transcription terminator	[22]	Up to 94-fold dynamic range
tna adapter	Transcriptional regulation via translational regulation of *tna* leader peptide synthesis	[88]	10^3-fold dynamic range when combined with the IS10 translational regulator
CRISPR-dCas9 transcriptional regulation			
dCas9 transcription repression	Repression of transcription initiation or elongation	[19,33]	Programmable sgRNA directs dCas9 to specific sequences for 10^3-fold repression
dCas9:RNAP ω transcription activation	Recruitment of RNAP ω subunit to promoter enhances transcription initiation	[33]	Largest impact observed with weak promoters
RNA degradation			
Csy4 cleavage	Csy4 endoRNase degrades target mRNA	[16]	Requires the Csy4 target sequence to be encoded within the open reading frame
Self-cleaving aptazyme	Ligand-responsive ribozyme encoded at the 3′ end of mRNA	[105]	Modular design can incorporate different aptamers
Control of protein levels			
RBSs	Control of translation initiation rate	[110–112,116,117]	*In silico* modelling has good predictive power
Orthogonal ribosomes	Modified 16S rRNA initiates translation only from cognate orthogonal mRNAs	[118,119]	Three additional orthogonal ribosomes are available
Riboswitches	mRNA secondary structure occludes the RBS in a ligand-dependent manner	[121–123]	A limited number of ligands can be sensed; temperature sensing is also possible

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

Table 1 (*continued*)

Tool family	Action	References	Notes
taRNAs			
sRNAs	Antisense RNA binds mRNA to block RBS and promote degradation	[124–126]	Conceptually simple to design, can target native mRNAs
IS10 repression	taRNA binds to *cis*-region of mRNA to block RBS	[18]	Many sets containing two to seven orthogonal pairs exist; requires *cis*-element upstream of open reading frame
Toehold switches	Trigger RNA sequesters a branch of a translation inhibition hairpin in the mRNA	[20]	Dynamic range of up to 600-fold activation; very few constraints trigger/switch binding sequence
Protein degradation			
(Inducible) ssrA-tagged degradation	SsrA tags target protein for degradation by ClpXP machinery	[132,134,135]	SspB chaperone activity can be induced to tune degradation rate
M. florum Lon degradation	*M. florum* Lon protease is orthogonal to *E. coli*; modified tags can be recognised by both ClpXP and Lon	[136]	Various steady-state and inducible degradation rates available
Beyond transcription and translational control			
Protein splicing	Split intein used to form a peptide bond between two proteins	[144]	Two- and three-way splicing is possible
Protein cleaving	Can be used to release a sequestered factor	[148]	Example uses the tobacco etch virus protease
Protein scaffolds	Protein binding domains used to immobilise and organise enzymes	[150,24,154]	Stoichiometry and relative position on the scaffold can be controlled
RNA scaffolds	CRISPR guide RNAs or multi-dimensional RNA structures as scaffolds for protein binding	[101,155]	CRISPR guide RNAs can recruit proteins to DNA; multi-dimensional RNA scaffolds spatially organise metabolic pathways
Inducible association of proteins	Target proteins are fused to signal-responsive interacting domains	[134,156–158]	Interacting domains that respond to small molecules or light are available
DNA modification for memory			
Recombinases	Recombinases flip a section of DNA	[162–164]	Memory elements can be nested or concatenated; excisionases can be employed to reverse DNA flipping
Retron-encoded analogue memory	ssDNA produced in response to a signal is incorporated into the genome at a replication fork	[25]	DNA changes can be targeted to any unique sequence in the genome; recombination frequency is proportional to ssDNA expression level

ions are widely employed, though future complex gene circuits will require a larger number of inducible transcription regulators that are orthogonal to each other and the cell's metabolism (i.e., low toxicity, low cross-talk with endogenous regulators, unresponsive to endogenous metabolites). Whilst some existing protein regulators have been adapted to work in new contexts, for example, by rational modification of the DNA binding specificity [65] or constructing chimeric proteins through the fusion of different sensing and DNA binding domains [66], the variation in natural mechanisms of inducer binding and conformational change makes scalable, modular, and predictive design of new regulator proteins difficult (RNA sensors of small molecules will be discussed later). For example, computational redesign of the QacR binding pocket was recently used to expedite an *in vitro* screen of new transcriptional regulators responsive to vanillin, but the best variants had a low dynamic range (ON:OFF ratio less than 10) [67].

In the absence of a characterised sensing system for a metabolite of interest, it may be possible to find native promoters that respond to the molecule via global transcript profiling. Dahl *et al.* pioneered this approach, using microarrays to identify promoters that are differentially activated in response to the toxic intermediate farnesyl pyrophosphate in the isoprenoid biosynthesis pathway [68].

Small-molecule control: Riboswitches

Small-molecule control over transcription can also be achieved using riboswitches. These structures in the 5′ UTR of an mRNA contain a regulatory domain that undergoes restructuring upon ligand binding to an adjacent aptamer domain. The resulting structural change may either repress or activate transcription [27,69]. *Cis*-elements that control the formation of a transcription terminator hairpin have been demonstrated in *E. coli* that are responsive to, for example,

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

theophylline [70], guanine [71], and *S*-adenosylmethionine [72], albeit with fold changes of less than two orders of magnitude. Riboswitch engineering has produced aptamers that sense unnatural compounds that are orthogonal to the cell's metabolism [73,74], though the variety of ligands sensed by riboswitches is limited in general [69]. *In vitro* screening and selection methods for aptamers with novel binding properties are well established [75], but further development of complementary experimental [76] and rational design [71,72] strategies for transferring these properties into effective *in vivo* riboswitches is required [77].

Liu *et al.* demonstrated a scalable design for small-molecule control of transcription, using unnatural amino acid (UAA) concentration to either positively or negatively regulate the transcription of

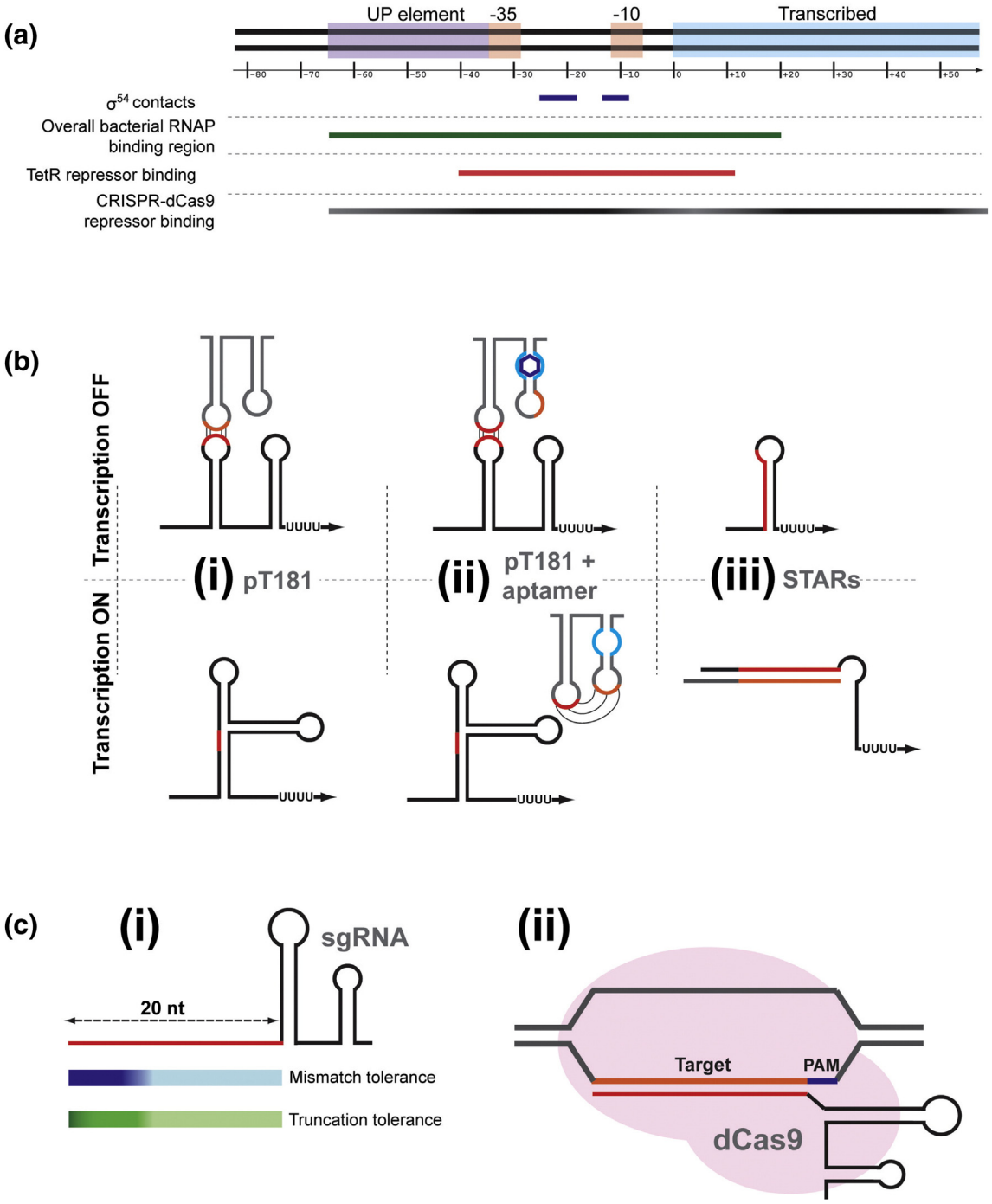

Fig. 1 (*legend on next page*)

downstream genes [78]. Both activating and repressing control regions feature a leader peptide gene containing UAA codons; if the corresponding UAA-charged tRNA is absent, the ribosome will stall. The positively regulated design, based on the *tna* operon, then features a Rho transcription termination sequence ahead of then the regulated genes; if the leader peptide is translated without ribosome stalling (UAAs available), the Rho binding site is blocked, and translation of downstream genes occurs. The negatively regulated design, based on the *trp* operon attenuator, replaces the Rho binding site with a structured RNA region that forms a transcription termination hairpin if the leader peptide is translated without stalling. These designs are scalable, being programmed by the type of UAA incorporated in the leader peptide, and possess 10- to 100-fold dynamic ranges in their output.

Optogenetics

Light is an orthogonal signalling medium for many bacteria and is of particular interest for industrial applications where the addition of a chemical inducer is expensive or would contaminate the product. Red-light-sensing and green-light-sensing transcriptional activating systems that can work in tandem have been engineered, using the chimeric Cph1-EnvZ/OmpR (red) [79,80] and the unmodified CcaS/R (green) [81] two-component histidine kinase signalling pathways. Additionally, the blue-light-responsive YF1/FixJ two-component system has been engineered into light-activating and light-repressing configurations [82].

RNA control of transcription

A major strength of RNA-based parts for gene circuits is that they are amenable to *in silico* design: thermodynamic models are able to make good predictions of the secondary structure of, and interactions between, small *trans*-acting RNAs (taRNAs) and their cognate *cis*-elements [27,83,84]. The simple composition of RNA aids design but is also a relative weakness: proteins are more chemically diverse and consequently may be able to interact with a more diverse set of ligands and with a greater span of binding affinities [27]. Protein-based regulators generally exhibit greater dynamic ranges and stronger levels of repression and have longer half-lives [27]. However, the larger sequence and chemical diversity of proteins makes their interactions much more difficult to model compared to RNA; consequently, RNA-based part families are likely to be preferred for the construction of complex large gene circuits in the near future [27,84].

With regard to transcriptional control, a number of variants of the pT181 transcription attenuation system have been developed for gene circuit control in *E. coli*: the native pT181 mechanism uses a structured 5′ UTR that forms a hairpin, normally allowing transcription elongation; a "kissing loop" interaction of a hairpin on the taRNA with the 5′ UTR hairpin promotes the formation of a downstream transcription terminator stem–loop structure [85] (Fig. 1b-i). Lucks and co-workers altered the terminator stem of the native system to increase the dynamic range of repression to five-fold and created three orthogonal hairpin/taRNA pairs by mutating the loop of the control hairpin [85]. Another 11 pairs were created in a later study using chimeras with hairpin elements from taRNA-controlled translational regulators [86]. Multiple regulators can be placed upstream of a coding region to create NOR logic or improve repression [85]. A further adaptation of the system introduced a small-molecule-sensing aptamer to the taRNA that interferes with the interacting hairpin in the ligand-unbound state; binding of the ligand frees the taRNA hairpin to interact with its cognate loop on the 5′ UTR hairpin region and inhibit translation [87] (Fig. 1b-ii). The design is modular and able to integrate different aptamers as long as the structure is known [87].

Recent work on the pT181 system has adapted it to enable taRNA activation of transcription, by changing the default secondary conformation to a stable transcription terminator. Small transcription activating RNAs (STARs) have been engineered to disrupt the terminator, either indirectly via interaction with an upstream hairpin structure or through direct

Fig. 1. Tools for engineering transcriptional control. (a) Key regions for transcriptional control at a promoter. RNAP binds the promoter via σ^{70} interactions with the −10 and −35 regions; additional interactions take place between the α subunit C-terminal domains and the UP element. Binding regions at −12 and −24 for the alternative σ^{54} factor are indicated below. Effective TetR repression requires disruption of transcription initiation by preventing RNAP binding and open complex formation [21]. CRISPR-dCas9 can block transcription initiation or elongation—repression is most effective when preventing σ factor binding or blocking RNAP progressing at the start of the transcript (grey line, darker colour indicates more effective repression) [19,33]. (b) Control of transcription by taRNAs: (b-i) the native pT181 *cis*-element allows transcription by default; the presence of a cognate taRNA induces the formation of a transcription terminator hairpin; (b-ii) the pT181 taRNA can be engineered to contain an aptamer—ligand binding releases internal pseudoknot interactions, allowing taRNA binding to the *cis*-element and repression of transcription [87]; (b-iii) direct-acting STARs sequester one branch of a terminator hairpin, activating transcription. (c) Features of CRISPR-dCas9 transcription repression. (c-i) The 20 nucleotides at the 5′ end of the sgRNA (red) is responsible for DNA binding; the first eight nucleotides are not essential for specificity (blue bar, darker indicates more tolerant); a guide length of 20 nucleotides is optimal—repression is negligible using 11 nucleotides or fewer (green bar, darker is more tolerant). (c-ii) DNA target (orange) binding by the sgRNA-dCas9 complex requires initial recognition of the adjacent PAM sequence (purple) in the DNA.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

sequestration of one branch of the terminator stem [22] (Fig. 1b-iii). This positive regulation configuration of the pT181 system exhibits a higher dynamic range (94-fold) compared to the repressor and is equally amenable to concatenation for the creation of layered logic gates.

taRNAs have also been incorporated into a modular regulator based on the previously described regulation of transcription via control over *tna* leader peptide translation [88]. The leader peptide functions as a modular "adapter", allowing a number of both negative and positive translation regulation mechanisms to be used to control transcription, including the IS10 RNA-IN/RNA-OUT motif [18] that allows for nearly three orders of magnitude of transcriptional repression [88]. Multiple *tna* adapter units can be combined to control a single transcriptional output, though Csy4 processing is required to allow proper folding of RNA-IN *cis*-elements.

CRISPR-dCas9 control of transcription

Clustered regularly interspaced short palindromic repeat (CRISPR) systems recognise, remember, and destroy foreign nucleic acids, acting as immune systems in their hosts to defend against invading phage and plasmids [27,55,89]. Natural CRISPR systems comprise a set of DNA-encoded target sequences—the CRISPR array—which are transcribed and processed into guide RNAs. These RNAs target CRISPR-associated proteins to complementary sequences that are cleaved, allowing the specificity of CRISPR-associated protein binding to be programmed by modifying the sequence of the guide RNA. It is this function that makes adapted CRISPR systems such attractive tools for genetic engineering. The Cas9 protein from the *Streptococcus pyogenes* type II CRISPR system has been widely applied as a minimal functional unit for the recognition and cleavage of target double-stranded DNA for genome engineering [90–94] and in its mutant endonuclease-inactivated form dCas9 as a DNA binding protein [19,33]. Cas9 requires both a guide RNA and a tracrRNA (*trans*-activating CRISPR RNA) to function; the guide RNA can be expressed either as a crRNA (CRISPR RNA) as it is from the CRISPR array or as a fusion to the tracrRNA known as an sgRNA (small guide RNA) [95] (Fig. 1c-i). Target sequences must be directly downstream of a three-nucleotide "NGG" protospacer-adjacent motif (PAM) for recognition and cleavage to occur [96] (Fig. 1c-ii). Two recent efforts have produced split versions of Cas9 to achieve increased functionality: reformation of the functional enzyme can be mediated by small-molecule binding for rapid induction [97] or by using the sgRNA as a scaffold [98]. Here we will consider the use of dCas9 in bacterial transcriptional regulation.

CRISPR-based repression of transcription uses dCas9 to block RNAP access to a promoter or prevent transcription elongation—a property that most other repression mechanisms do not have, allowing transcription of downstream genes in a polycistronic unit to be selectively knocked down. The highest repression is achieved by targeting dCas9 to the transcription initiation region (especially the −35 box [19,33]) or the antisense strand close to the start of a target gene if preventing transcription elongation [19]. Repression is highly effective, up to 1000-fold when targeting a gene with two sgRNAs [19], and tuning of repression is also possible though the introduction of mismatches between the guide and target sequences or truncation of the guide [19,33]. The 12 bases of the guide RNA adjacent to the PAM are the minimal requirement for specificity [19], making it unlikely that off-target binding will occur in bacterial genomes [27]. Expression of multiple guide RNAs enables parallel control over multiple genes [19,99,100]. Guide RNAs can be expressed as a series of sgRNA transcriptional units: Nielsen and Voigt used Golden Gate assembly to combine multiple units for the construction of a gene circuit with three layers of sgRNA control [100]. Alternatively, Cress *et al.* employed type IIS restriction enzyme assembly for the construction of a synthetic array of crRNAs transcribed from one promoter, which was used to simultaneously repress three endogenous *E. coli* transcripts [99].

Transcription activation is also possible by fusing dCas9 to an activator protein. The only example so far in *E. coli* uses a fusion to the RNAP ω subunit, with dCas9 targeted approximately 90 base pairs upstream of the transcription start site [33]. Activation is modest (23-fold for a weak promoter, lower effects for stronger promoters) and requires a Δ*rpoZ* strain but might be improved through fusions with (multiple) alternative factors. Rather than using a direct fusion to dCas9, the guide RNA can be also used as a scaffold for hairpin binding proteins, which are themselves fused to an effector protein, enabling recruitment of different effectors to different sites [101].

RNA degradation

Targeted cleavage of mRNA molecules was demonstrated by Qi *et al.* who used and inserted an in-frame cognate sequence for the *Pseudomonas aeruginosa* CRISPR endoRNase Csy4 immediately downstream of the start codon, resulting in better than 10-fold repression of protein expression [16]. This method obviously is constrained by having to integrate the Csy4 target sequence into an open reading frame, but other CRISPR-based technologies may enable specific, programmable degradation of mRNA in the future: Mitchell and co-workers showed that, *in vitro*, the type II Cas9 endonuclease can be targeted to and cleave single-stranded RNAs by supplying the PAM motif *in trans* on a single-stranded DNA (ssDNA)

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

oligonucleotide [102]. As pointed out by Nelles *et al.*, it has not been shown that this technique would work *in vivo* or that cleavage of DNA would not also occur [103]. The targeted RNA binding function of nuclease-inactivated Cas9 (RCas9) could still be very useful, and Nelles *et al.* suggest other potential applications for RCas9, including targeting proteins to an RNA that then acts as a scaffold, combining sensing with actuation [103]. Other CRISPR systems also show potential as programmable bacterial RNAi tools, for example, the *Streptococcus thermophilus* type III-A Csm complex, which was recently shown to preferentially cleave single-stranded RNA *in vitro* and repressed replication of the MS2 RNA phage when the components were expressed in *E. coli* [104]. *Trans*-acting elements are not necessarily required: Win and Smolke introduced a ligand-regulated self-cleaving ribozyme (aptazyme) into the 3′ end of an mRNA to control gene expression, using a modular design that can use different aptamer domains [105]; ligand binding could either disrupt or reinforce the ribozyme fold, modulating the self-cleaving activity.

RNA stability can be improved by protecting long single-stranded regions from RNase E degradation by introducing hairpin secondary structures [106,107]; RNA half-life in *E. coli* correlates somewhat with the folding energy of 5′ hairpins, indicating that this protection against RNase E is an important, but not exclusive, factor for RNA stability [108]. Stronger ribosome binding site (RBS) sequences also impart a small improvement in mRNA stability, presumably due to the bound ribosome blocking RNase access [40].

Tools for Engineering Protein Levels

In contrast to the multiple routes for regulating transcription, peptide synthesis rates are mostly accessible to regulation by RNA—both the mRNA template itself and taRNAs. Translation can begin at internal sites in the mRNA, allowing control over the initiation rates of individual reading frames within a polycistronic transcript.

Translation initiation control with designer RBSs

Translation initiation requires recognition of an RBS by the 30S ribosomal subunit, through base-pairing between the RBS and an anti-Shine–Dalgarno sequence encoded in the 16S ribosomal RNA (rRNA). The strength of the mRNA-rRNA interaction, as well as accessibility of the region to the ribosome, determines the likelihood of the translation complex forming and is an important control point for gene expression [109] (Fig. 2a). Thermodynamic models of translation initiation have been developed by a number of groups, which all show good predictive power [110–112]. These models have been implemented in computational tools that enable efficient sampling of a large range of translation initiation rates, allowing optimisation of protein expression levels in a synthetic biological system [107,113]. Farasat *et al.* demonstrated effective sampling of expression levels in different bacteria and also combined kinetic modelling of a biosynthetic pathway (which includes enzyme expression parameters) with translation initiation rate prediction to find variants with improved productivity [107]. A complementary approach using directed evolution rather than pathway modelling was employed in another study, combining RBS library design with the multiplex automated genome engineering (MAGE) recombineering method [114] to optimise a five-gene pathway, using 16 RBS variants for each gene that sampled a range of translation initiation rates spanning three orders of magnitude [115]. These translation initiation rate prediction tools are available online, for example, the RBS Calculator [116], which has been recently updated to incorporate parameters developed by Borujeni *et al.* [117].

Orthogonal ribosomes

Ribosome recognition of the RBS in an mRNA occurs through base-pairing between the RBS and an anti-Shine–Dalgarno sequence encoded in the 16S rRNA, a fact that has been exploited to generate three additional orthogonal ribosomes with modified 16S rRNA that initiate translation only from mRNAs with cognate orthogonal RBSs [118]. Transcription of both the orthogonal rRNA and mRNA is required for gene expression, creating AND logic for greater control over the output [119]; the orthogonal rRNA could be considered a *trans*-activating RNA in this particular context. A practical consequence of the use of orthogonal rRNAs is that the coding sequence of target mRNAs may need to be reconfigured to prevent pausing at Shine–Dalgarno-like sequences [120].

Riboswitches

Riboswitch control over translation occurs through ligand-dependent restructuring of the 5′ UTR of an mRNA to modulate access of the ribosome to the RBS [27,69]. As was discussed for transcriptional riboswitches, the range of ligands and the fold response are limited, but translational riboswitches have been employed successfully in synthetic microbial gene circuits, for example, to control chemotaxis [121]. Sharma *et al.* demonstrated that translation-controlling riboswitches can be combined to allow AND and NAND logic, though the composition required screening of variants (i.e., was not modular) [122]. Temperature-sensitive riboregulators of transcription (RNA thermometers) also act through control of RBS availability; an RBS occluded within secondary structures formed at low temperature becomes accessible at higher temperatures as the RNA melts [123].

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

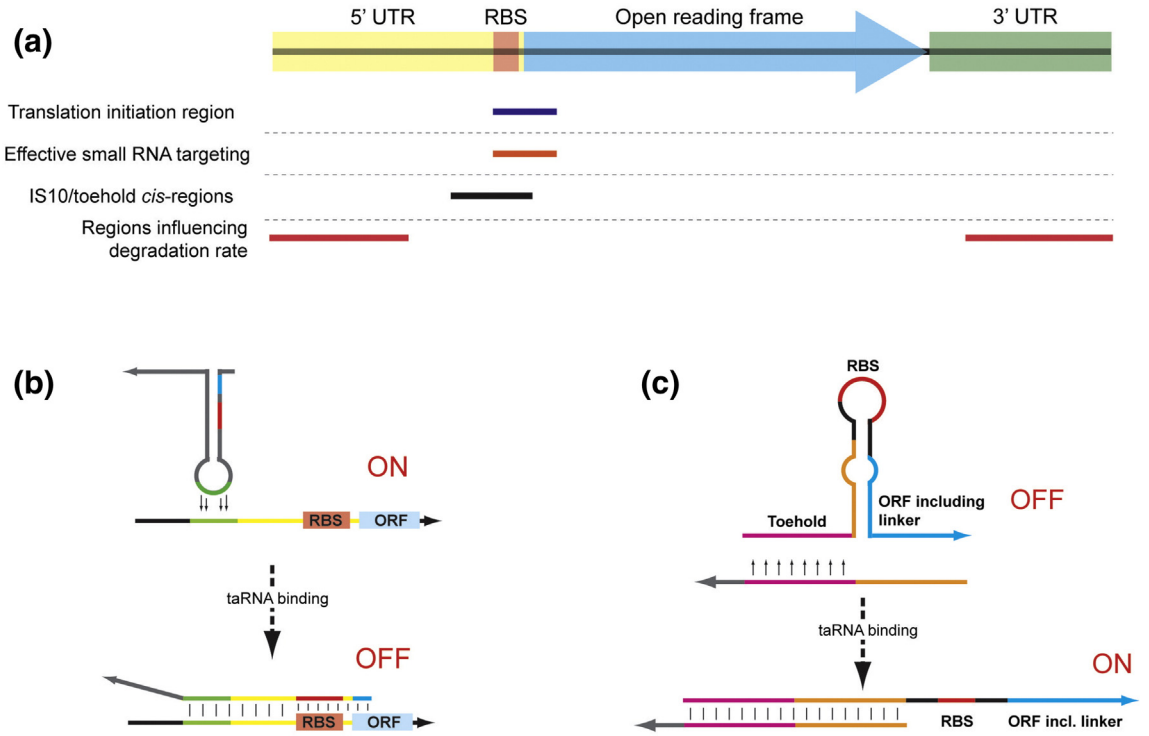

Fig. 2. Tools for engineering translational control. (a) Key regions for translational control on an mRNA: the ribosome interacts with the translation initiation region via interactions between the 16S rRNA and the RBS; sRNA repression is most effective when targeting this region, and the IS10 and toehold switch *cis*-elements function by sequestering the RBS and start of the open reading frame (ORF) [18,20]. The structure of the 5′ and 3′ ends alters accessibility to RNases. (b) IS10-based translational repression: binding of the taRNA (RNA-OUT) and resultant occlusion of the RBS is initiated via interactions between its specificity loop (green) and the cognate sequence in the RNA-IN *cis*-element. (c) The toehold switch *cis*-element sequesters the RBS (red) and ORF start (blue) in a hairpin. Binding of the trigger taRNA initiates using the toehold region (purple) and displaces the hairpin to also bind the 5′ branch (orange), freeing the RBS.

taRNA control of translation

Trans-repressing RNA control

The mechanism of translation inhibition by small RNAs (sRNAs) is conceptually simple, making this method easy to implement: the sRNA contains a complementary sequence to the target mRNA, binding at or just downstream of the RBS to block the ribosome (Fig. 2a), with sRNA-mRNA binding energy correlated to repression strength [124]. In addition to the targeting region, a 3′ scaffold recruits the Hfq chaperone protein that stabilises the sRNA-mRNA interaction and promotes their degradation by RNase E [125]. Synthetic sRNAs that use the scaffold portion from an endogenous *E. coli* sRNA [124,126] with a designed 5′ antisense region may be designed: Na and colleagues used the MicC scaffold to make over 120 sRNAs, allowing them to repress multiple target metabolic genes and screen for increased productivity of a desirable compound [124]. Endogenous sRNAs target a range of locations on target mRNAs (i.e., not always the RBS) using a variable number of bases [126]; thus, the strategy employed by Na *et al.* of screening multiple sites on and around the translation initiation region of the target mRNA for effective repression may be advisable [124].

The native IS10 translation control system is composed of a taRNA (RNA-OUT) and a cognate RNA-IN sequence surrounding the RBS and start codon of the controlled mRNA. The free antisense RNA-OUT molecule contains an interacting hairpin, the loop of which initiates binding to the RNA-IN sequence to block ribosome access to the translation initiation region [18] (Fig. 2b). The loop region defines the specificity of the interaction between RNA-IN/RNA-OUT pairs, and Mutalik *et al.* manipulated the sequence of the loop in a streamlined version of the native RNA-OUT and screened a group of 23 variants for repression efficacy and orthogonality, then validating an orthogonal set of five *in vivo*. Data from the experimental group were used to predict the existence of thousands of sets of pairs (sets containing two to seven members) that exhibit less than 20% cross-talk.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

The IS10 system has also been adapted for small-molecule sensing by adding an aptamer to the RNA-OUT sequence [87].

Trans-activating RNA control

Green *et al.* recently described the development of toehold switches, translation-blocking hairpins that sequester the RBS and start codon of a gene, with translation activation occurring through binding of a short (30–40 bases) trigger RNA that sequesters the upstream branch of the hairpin (similar to the mechanism of direct STAR transcription activation) [20] (Fig. 2c). Binding initially occurs to a single-stranded region 5′ of the hairpin (the "toehold"), and the hairpin is designed so that only the RBS and start codon need remain constant between different switches: the start codon is part of the 3′ branch of the hairpin, meaning a seven-amino-acid linker is added to the protein. Whilst alternative efforts to develop *trans*-activating RNA systems do not alter the coding of the regulated gene [127–129], the toehold switch design places very few constraints on the sequence when constructing trigger-switch pairs, potentially allowing the creation of a huge number of orthogonal pairs. The most effective toehold switches have a dynamic range of over 600-fold, an order of magnitude better than most other taRNA control mechanisms. Green *et al.* showed that multiplexed control over the translation of four fluorescent proteins transcribed as a single polycistronic mRNA is possible with minimal polar effects, demonstrating the potential for sophisticated control over synthetic gene networks. Endogenous genes can also be placed under the control of trigger RNAs by altering the chromosomal copy of the gene to introduce the regulatory hairpin, but this intervention could disrupt native regulation (of transcription as well as translation) [130]. Another application is to sense RNAs—the RNA being sensed is used as the trigger—which has been shown to work in cell-free, paper-based devices for sensing Ebola mRNA [131].

Protein degradation rate tuning

The concentration of a protein and dynamics of its expression may also be controlled via manipulation of its degradation rate. SsrA degradation tags of varying strength [132] are available, though these make use of host ClpXP machinery—a fact utilised by Prindle *et al.* to temporally coordinate protein expression with delay times of less than a minute [133]. Inducible degradation of proteins is also possible: SspB-dependent chaperoning of DAS-ssrA-tagged proteins for ClpXP degradation was made inducible by splitting SspB and fusing each half to FRB or FKBP12, two proteins that are brought together through binding rapamycin [134]; replacement of the native *sspB* promoter with an anhydrotetracycline-inducible promoter has also been demonstrated [135]. More recently, a hybrid system that can degrade proteins using either the native *E. coli* ClpXP or an inducible orthogonal Lon protease from *Mesoplasma florum* has been developed [136]. The 27-amino-acid *M. florum* ssrA tag was modified at the C-terminus to create a set of variants that are recognised by the endogenous *E. coli* degradation machinery to give a range of steady-state levels of a target protein whilst remaining an effective signal for *M. florum* Lon degradation. The efficacy of *M. florum* Lon degradation could also be modulated through variation of residues 13–15, creating a library of tags with a range of steady-state and inducible degradation rates.

Beyond Transcriptional and Translational Control

In addition to the regulation of RNA and protein synthesis, biological signal modulation also occurs through the interactions between and within these molecule classes (e.g., binding [32,45], chemical modification [137]), providing additional opportunities for control. Compared to transcription and translation, information transfer between interacting molecules can be extremely rapid and resource efficient [138].

Modulation of transfer functions

The HrpRSV system contains examples of protein/protein interactions that both amplify and diminish a signal: HrpR and HrpS form a heterohexamer that activates σ^{54}-dependent transcription from the P_{hrpL} promoter (allowing AND logic); inhibition of HrpS by HrpV negatively modulates the output from P_{hrpL}, reducing the gain and dynamic range of the output [45,139] (Fig. 3a). The *in trans* inhibition by HrpV illustrates an important general strategy for lowering the OFF level of a signal and increasing the sensitivity of a response—a key property in biological circuits that require digital characteristics or bistability [11,26,137,140]. Briefly, *in trans* inhibition or sequestration may be designed into a gene circuit, for example, by introducing additional decoy binding sites for transcription factors [141], expressing specific sequestering proteins [32,45] or antisense RNAs [142]. Positive cooperativity also increases response sensitivity, occurring when ligand binding increases the binding affinity for subsequent ligands [143].

Splicing and cleaving

Splicing and cleavage reactions can be used for rapid, stable switching of function. Split inteins have been employed in a number of contexts for protein splicing, usually to reconstitute an active enzyme from two (or more [144]) inactive components [23,97,98] (Fig. 3b-i). RNA splicing also occurs in bacteria [145–147], though this function has not been adapted for use in synthetic gene circuits *in vivo* to the best of our

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

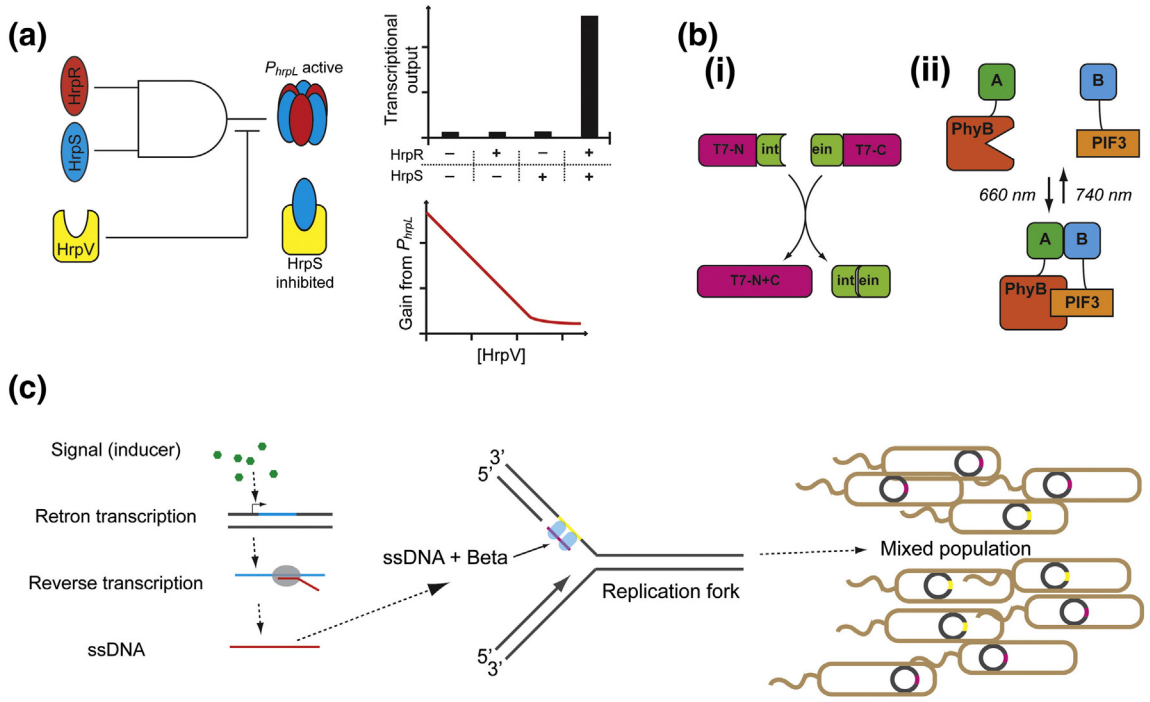

Fig. 3. Beyond transcriptional and translational control. (a) The HrpRS proteins combine to activate transcription from the P_{hrpL} σ^{54} promoter, enabling AND logic functions (upper chart). Inhibition of HrpS by HrpV modulates the transfer function, allowing the transcriptional amplification gain from P_{hrpL} to be tuned in an analogue fashion (lower chart) [45]. (b) Control of protein/protein interactions: (b-i) split T7 RNAP (pink) can be stably reconstituted using split inteins (green); excision of the intein results in concomitant splicing of the N- and C-terminal T7 RNAP fragments [23]. (b-ii) The interaction between proteins A (green) and B (blue) can be mediated by reversible light-dependent binding between PhyB and PIF3 [158]. (c) Bacterial DNA can be altered *in vivo* in a targeted fashion using Beta recombinase to facilitate integration of mutation-containing ssDNAs (red) to a homologous sequence within a replication fork (yellow) [25]. The signal is recorded across the population, with the number of mutants proportional to signal intensity or duration.

knowledge. Cleavage can be exploited for inactivation of a component, for example, RNA aptazyme-mediated self-cleaving [105], but it has also been used to activate signalling by releasing a sequestered transcription factor [148].

Scaffolds

An alternative to covalent fusing of components is to stabilise their interactions using a scaffold, enabling modular, predictable, and scalable organisation of substrate components. Protein scaffolds have been adapted to re-wire yeast signalling pathways [149] and built *de novo* to sense and control protein concentration [150]. There is much potential in using scaffolds (and micro-compartments [151,152]) to coordinate metabolic pathway enzymes: enzymes and intermediates are concentrated to minimise diffusion times of unstable intermediates [153], and the stoichiometry and position of components on the scaffold can be easily manipulated [24,154]. RNA scaffolds can be used in isolation, for example, CRISPR guide RNAs that act as scaffolds for transcriptional effectors [101], but they can also be engineered to produce sophisticated higher-order structures [130]: Delebecque *et al.* made use of two-dimensional RNA scaffolds to improve hydrogen production in *E. coli* [155].

Inducible association

Small-molecule-induced association of proteins can be engineered by fusing the target proteins to interacting domains, for example, FRB and FKBP12 for rapamycin-dependent binding [134]. Photo-modulation of protein/protein interactions is also possible: the PhyB/PIF3 (phytochrome) and Cry2/CIP1 (cryptochrome) pairs respectively exhibit red-light-dependent and blue-light-dependent association and have been fused to DNA binding and activation proteins to enable photo-activation of transcription in yeast [156–158] (Fig. 3b-ii); these systems could also be exploited in bacteria, for example, to accelerate split-intein-based reconstitution of transcription factors [156].

DNA modification for memory

Memory can be built into gene circuits using network motifs that generate robust bistability [159–161], but

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

recent developments now allow direct editing of the organism's native data storage medium, DNA. Recombinases can be used to excise or flip a section of DNA, depending on the orientation of flanking recombination sites; incorporation of a control sequence such as a promoter or terminator gives a read-out of the orientation of the memory element, and memory elements can be concatenated or nested to create different two-input logic gates [29,162]. Genome mining was recently applied to identify 11 orthogonal phage integrases, which were all used in the creation of a memory array that can record 2048 (2^{11}) unique states, as well as for the assembly of three-layer cascades [163]. This expansion in the size of available permanent biological memory could be directly beneficial to environmental sensing systems [159] and should facilitate the construction of novel synthetic bio-computation architectures [163]. Bonnet *et al.* also incorporated an excisionase into their system to allow the orientation of memory elements to be re-set, creating re-writable memory [164].

Recombinase-based memory is limited by the small number of orthogonal recombinases, but Farzadfard and Lu have described scalable DNA-encoded memory that is encoded in an analogue fashion across a population of cells [25]. ssDNA is produced via reverse transcription of a retron, which is itself transcribed from an inducible promoter in response to the input being recorded (e.g., presence of drug). ssDNAs are designed to be mostly homologous to the target locus but introduce a desired mutation, and cells are engineered to express Beta recombinase to catalyse incorporation of the ssDNA at a replication fork (Fig. 3c). The introduced mutation can be designed to cause loss or gain of function in a gene, allowing the population to be screened and memory to be read. The number of recombination events is proportional to the number of ssDNAs produced, that is, the concentration of inducer, and memory can be re-written by expressing a different ssDNA to target the same locus.

Principles for Robust and Scalable Gene Circuit Design—Modularity and Orthogonality

Despite having a large and growing resource of components, an inability to accurately predict how they will function when combined means synthetic biologists have not succeeded in producing corresponding increases in gene circuit size [42]. Modularity is the quality of consistent function in a variety of contexts and is a core requirement for predictive design of complex gene circuits [8,15,165]. The complementary property of orthogonality implies that parts and modules will not display undesirable cross-talk with other elements in the engineered biological systems, as well as with the host genetic background. Orthogonal parts and modules are important for the compatibility of parts within, and scalable design of, large gene circuits comprising many components [44]. Ideally, synthetic biologists would like the components they use to be "plug and play", functioning reproducibly in different circuit designs; in practice, the complexity of *in vivo* biological systems makes this very difficult to achieve. Unlike electronic digital circuits, the components in a biological circuit are not connected by insulated wires, and the flow of biological information has to depend on specific chemical interactions to avoid cross-talk. The interactions of gene circuit components with each other and with the host organism alter their function through unexpected contextual effects [14,15,166], including retroactivity—especially in larger circuits [137,165,167,168]. Here we introduce principles for building scalable gene circuits, discussing how context effects arise and how they can be mitigated, either through extensive characterisation of the gene circuit or through the insulation of components.

Physical composition

Undesirable contextual effects can occur through unanticipated interactions between the components of a gene circuit and between the gene circuit and the host environment [14,15].

Starting at the level of the physical assembly, parts can be sensitive to sequence changes at their boundaries [169–172]. From their analysis of 12,563 promoter/RBS combinations, Kosuri *et al.* found that nearly 17% of the variation in protein levels could not be predicted from the promoter or RBS strength [40]. A conceptual solution to the problems arising from physical composition is to expand the definition of a part to include the regions that influence its function. Informed by related work that identified the promoter:UTR and UTR:gene of interest (GOI) junctions as important sources of variability in gene expression [44,166] (Fig. 4a-i), Mutalik and coworkers created an expression operating unit (EOU) [17]. The EOU comprises a standardised promoter sequence and 5′ UTR sequence adjacent to the transcription start site to remove context effects on transcription initiation, whilst a leader peptide that is translationally coupled to the GOI removes secondary structure around the GOI's RBS (Fig. 4a-ii). Expression can be tuned by varying the −10 and −35 regions of the promoter and the RBS for the GOI (encoded within the leader peptide sequence) and by introducing rare codons into the leader peptide. Use of the EOU imparts a high level of consistency to relative expression levels of a GOI when varying promoter and RBS sequences, enabling highly predictive design and tuning of protein levels in a gene circuit. Further improvements might include standardisation of the length of the transcription control element to include the UP element [8,38].

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

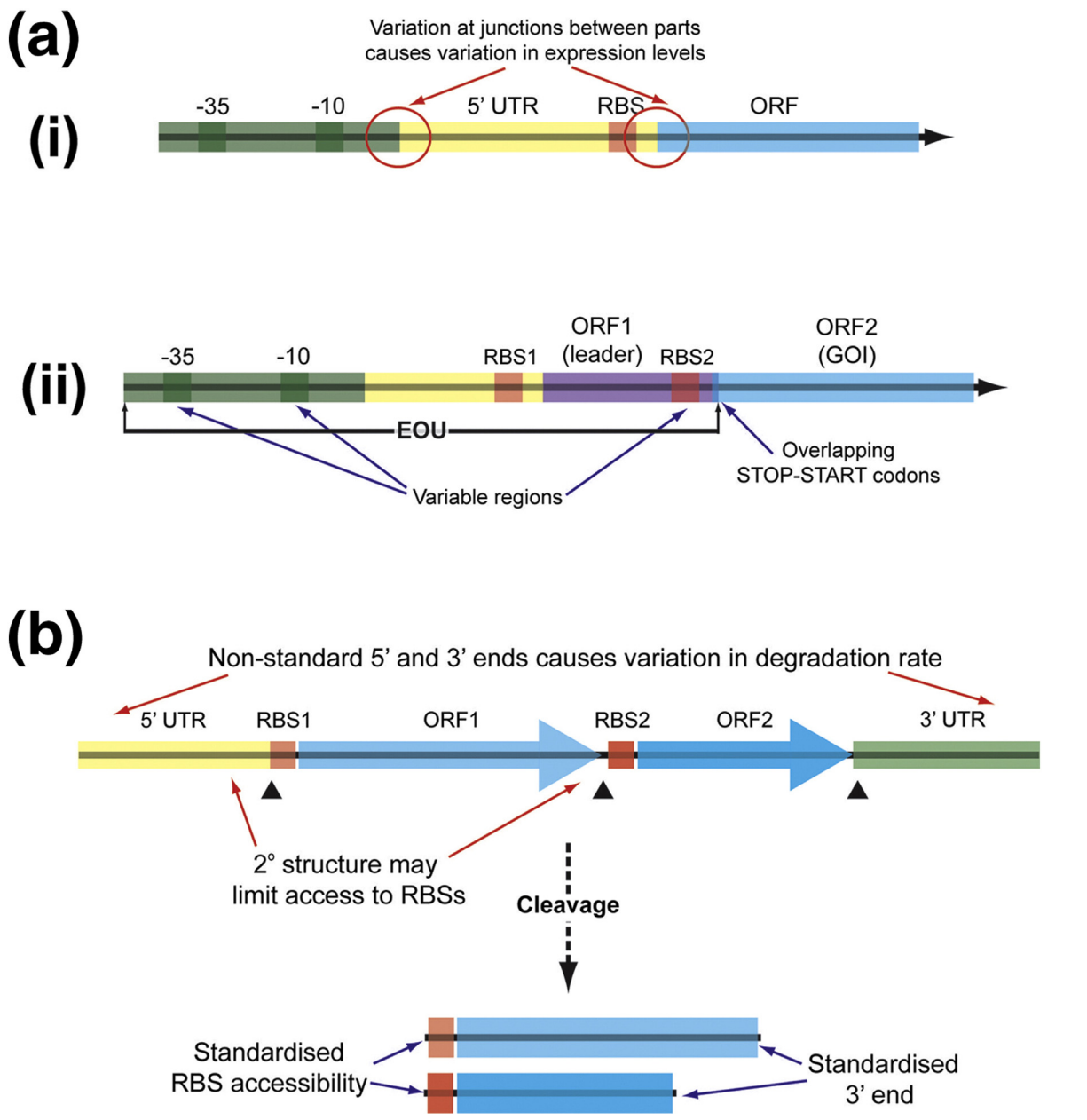

Fig. 4. Tools for improving modularity of gene circuit building blocks. (a) Reducing sequence context effects: (a-i) building new functional units thorough the combination of open reading frames and transcription and translation initiation parts results in variation at the junctions that can alter part activity. (a-ii) EOU uses translational coupling between a leader polypeptide and the GOI to reduce context effects. The sequence is fixed, except at the −10 and −35 regions of the promoter and the GOI's RBS (located within the leader ORF), to allow tuning of transcription and translation initiation rates. (b) Messenger RNA secondary structure can cause variation in protein expression levels from individual ORF in different transcriptional contexts. Cleaving the mRNA into minimal RBS ORF units (e.g., using Csy4 [16]; cleavage sites encoded at the black triangles) standardises ribosome access and RNA degradation rate.

Another approach to improve modularity is to insulate parts from their sequence contexts. At the DNA level, this might include adding standardised spacers between components [38] or preventing transcriptional read-through using transcription terminators. The high transcription rates of genes driven by T7 promoters are often a source of toxicity for the cell, making effective termination especially important in these cases [28,49]. There is the added benefit of producing defined mRNAs that possess a uniform 3′ structure and have a more consistent range of stabilities. Chen *et al.* created a useful resource through the characterisation of 582 terminators of varying strength, including a subset of 39

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

that have better than 98% termination efficiency and have enough sequence variation to prevent homologous recombination [173]. Improvements in the modelling of terminators will aid predictive design of effective, compatible sequences [174].

Post-transcriptional processing can also be employed to insulate RNA-based parts from their transcriptional context. It is often desirable to arrange genes in polycistronic units to reduce the size of constructs and avoid re-using promoter sequences. Qi *et al.* showed not only how moving monocistronic protein coding genes into the context of an operon caused variation (i.e., variably decreased) in the production of the encoded proteins but also that this effect could be countered using post-transcriptional modification of the mRNA to create monocistronic units [16]. Target sequences for the *P. aeruginosa* Csy4 endoRNase were inserted to flank the RBS and open reading frame, removing 5′ and 3′ UTRs, resulting in consistent and often increased expression levels of the proteins; access for the ribosome to the RBS is standardised, and variable 3′ sequences that influence RNA stability are excised (Fig. 4b). Lou *et al.* demonstrated a similar approach using a ribozyme encoded in the transcript to cleave the 5′ UTR [30], whilst 3′ RNase III sites decreased expression variability in the study of Cambray *et al.* [174]. Transcript processing also allows more complex sRNA regulation to be employed by insulating *cis*-regulatory elements from interactions with each other so that multiple regulatory elements can be designed into one polycistronic transcript [88]—spacers are not effective insulators of RNA *cis*-elements [85].

Functional composition

When parts are connected to form a circuit, failure may occur because the output range of an upstream component does not match the required input range of the downstream one. Components that have been characterised in isolation are functionally connected in a gene circuit, and their interaction alters the behaviour of the upstream component because of increased load [165]. This effect has been termed retroactivity and can be illustrated with the example of a transcription factor that binds an operator site: whilst the expression level of the transcription factor may have been characterised when it is expressed in isolation, the connection of downstream operator sites reduces the levels of available protein due to sequestration [167,175]. Recent work has demonstrated that it is possible to mitigate retroactive effects on signalling dynamics caused by increasing downstream load through the use of an insulating kinase/phosphorylase buffer module that has faster kinetics than the transcription-based components [137,168]. Otherwise, retroactivity must be corrected for through characterisation of parts in their functional context followed by tuning of the circuit [44].

Undesirable cross-talk between components may also occur. This effect can be mitigated by using well-characterised part families that have been shown to be orthogonal [18,21,32,45,50]. RNA-based part families have the advantage that cross-talk can be effectively screened for *in silico* using thermodynamic models [20]. Orthogonality is a key quality for the creation of scalable part families, allowing larger circuits to be constructed and creating a wider variety of alternative components for use when tuning a circuit.

Host context

When a gene circuit is propagated within a host organism, it competes against native host processes for cellular resources and can interact directly with host components; these effects can disrupt the normal function of both the gene circuit and the host.

Gene circuits use host machinery for activities including the replication of DNA, transcription and translation, and protein and RNA degradation, and they also draw energy and metabolites from host pathways. Cellular resource availability varies between growth conditions and host strains [176,177]. Cardinale *et al.* identified ribosome availability and growth rate as the key determinants for circuit performance across a range of *E. coli* strains, but they additionally found that specific deletions of host genes can also have a large effect [176]. These mutations indirectly effected circuit performance by altering flux through carbon and nitrogen metabolic pathways. The complementary approach of altering the host genetic (and metabolic) context through the enrichment of genes can also be beneficial for circuit behaviour [177].

If the burden of the gene circuit on the host exceeds homeostatic limits, the supply of resources needed for normal cell functioning and that of the gene circuit will be reduced, leading to altered behaviour or failure. For example, over-production of mRNA in *E. coli* causes competition for the translation machinery, leading to queuing that alters the dynamics of protein expression and upsets the correlation between mRNA and protein levels [178]. This overburdening can also cause a decrease in host protein production rates and inhibition of growth [179], effects that feed back on gene circuit function as altered expression levels and dilution rates. Similar effects occur if expression of heterologous proteins makes excessive use of rare codons, which can limit the overall rate of protein production due to a scarcity of charged tRNAs. Protein expression can be improved through codon optimisation [180], though the most optimal codon assignment may change depending on the state of the host [181]. Metabolic bottlenecks can also be purposefully utilised to exert control over global translation; Kobayashi [182] used a synthetic *gfp* gene (gene encoding green fluorescent protein) rich in rare

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

codons to suppress the global translation rate in *E. coli*, human cell lines, and adenovirus. As described previously for transcriptional control [78], this rare codon strategy might be combined with the use of UAAs: an open reading frame rich in re-purposed codons could be used as a general suppressor of genes that use those codons, or the total abundance of a set of synthetic proteins could be controlled though availability of the re-purposed tRNA. To prevent competition with the host for the transcription and translation machinery, the orthogonal alternatives of phage RNAPs [50] (possibly deployed within the previously described "resource allocator" framework [52]) and orthogonal ribosomes [118,119] can be employed. Further insulation from host processes might be possible in the future, by making use of reprogrammed or expanded genetic codes and UAAs [183,184].

Direct cross-talk between gene circuit and the host components can occur either through non-specific promiscuous interactions or through specific interactions that may cause interference [44] or toxicity [185]. Parts are often sourced from unrelated organisms in order to help avoid specific interaction between the circuit and host [44,136], and the genome can be screened for potential binding sites or interacting genes [19,21]—though these strategies cannot ensure that non-specific interactions do not occur. Non-specific interactions include off-target binding by protein/protein interaction domains [32] and transcriptional read-through [28]. The impact of part expression on the host growth rate and transcriptional profile can be quantified to aid selection of components that are orthogonal to the host [19,22,32]. Non-specific interactions are weaker than the desired specific interaction; thus, often toxic effects are only observed at high expression levels, leaving a reasonable functional range to work with [32].

Beyond orthogonality and modularity: Managing context effects for robustness and genetic stability

Robust gene circuits maintain their performance over time and across changing conditions—they respond adaptively to contextual perturbations such as host growth rate or transcription factor availability, and they often have a high signal-to-noise ratio, in order to produce consistent behaviour [186,187]. Gene circuits that function adaptively and do not over-burden their host are also more robust in terms of evolutionary stability [188]: as described previously, the burden imposed on a host by a gene circuit can reduce the growth rate, creating a selection pressure against that circuit. Gene circuits should be orthogonal to the host machinery wherever possible in order to reduce interactions with host processes and improve robustness. When interaction is unavoidable, the gene circuit can be designed to dynamically modulate its expression in response to increased strain on the host. An example of this is the negative feedback control of a biosynthetic pathway in response to elevated levels of a toxic intermediate [68], which improves the efficiency and productivity of the gene circuit, in addition to limiting the burden on the host. More complex regulatory networks might take inputs from a range of native promoters as a "fingerprint" to identify the metabolic state of the cell. In another example, Kushwaha *et al.* use integrated positive- and negative-feedback loops in their "portable expression resource" to tune the expression of T7 RNAP and maintain it at a non-toxic level in diverse Gram-positive and Gram-negative bacteria [189]. The study also shows the potential for engineering cross-species DNA parts, enabling the construction of genetic devices that function similarly across diverse organisms. Ceroni *et al.* recently used a "capacity monitor" to quantify the burden imparted by different gene circuit designs [190], concluding that the least burdened cells have more free ribosomes. This suggests that RNA-based circuits that perform analogue signal processing [11,191] are likely to be robust. The potential for evolutionary corruption of a gene circuit can also be reduced by not re-using parts, thus helping prevent recombination and choosing a host strain with reduced mutability [192].

Characterisation across contexts

It is clear that describing a part as modular also requires an understanding of the contextual limits of that modularity. Whilst insulation may preserve the properties of a component over a wider range of conditions, there will always be limits [193]. Beyond the contexts discussed above, wider environmental and ecosystem level effects—which can be controlled in the laboratory but are relevant to real-world applications—can interfere with circuit function and should be quantified [14,15]. The characterisation of parts under different conditions generates data that can be used to design an appropriate gene circuit for a given application [15].

As part libraries grow, the early calls for standardised descriptions of biological components become increasingly pertinent [194,195]. Common units such as polymerases per second and relative expression units facilitate comparison and exchange between engineers [9,39], and Mutalik *et al.* have outlined how the consistency of a component across contexts might be captured as a part "quality" statistic [166]. Olson *et al.* have produced an optogenetic system to drive gene expression for characterisation studies [196], whose rapidly reversible and easily tuned input allows interrogation of the dynamic response of a component [193]. These advances will hopefully improve our understanding of synthetic systems and accelerate progress towards their predictable design.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

Tools for Debugging and Tuning Gene Circuits

Without fully predictive design capabilities, the construction of a gene circuit inevitably requires cycles of debugging and tuning: the causes of failure must be identified so that the design can be adjusted to make the circuit operate within desired parameters, either by replacing a non-functional (e.g., toxic or having an irreparable impedance mismatch) component or by selecting a variant with a more appropriate transfer function. Here we set out methods for identifying and correcting defects in gene circuits and fine-tuning their characteristics, and we discuss DNA assembly strategies in the context of the refinement process.

Debugging—Identifying causes of failure

The change in function that can result from connecting a biological component into a gene circuit might be easily observed or inferred, especially in small circuits where there are few elements connecting a controllable input and measurable output. In such cases, proceeding directly to tune and reassess the circuit behaviour might be a more efficient strategy than characterising the activity of the parts in their new context. In a large circuit, the number of connections between input and output may obscure which components are performing poorly. For this reason, a good general rule is to build and test modules of a larger circuit before combining them [44] in order to characterise parts in a context that more closely resembles that of the complete system. Stepwise addition of parts or modules to a circuit, with characterisation of the resulting network and comparison to previous iterations, enables quantification of the effects of elaborating the module composition [193].

This characterisation strategy requires methods to assess the levels of internal (i.e., non-output) gene circuit components. Quantification of the relevant biomolecules using, for example, RNAseq, produces a snap-shot of the state of the circuit [40] and can also be used to assess the impact of the circuit on the expression of native genes [22]. In addition, synthetic biology tools in the form of sensors and reporters have been employed to investigate the state of gene circuit components. The activity of a promoter can be easily assessed by placing a reporter gene under its control; protein amounts [197] or localisation [198] can be monitored using a fusion to a reporter enzyme or scaffold [150], if the topology of the target protein permits. Similarly, RNA levels can be tracked *in vivo* through co-transcription with the Spinach aptamer [199], an RNA that activates fluorescence from its cognate fluorophore upon binding (a range of ligands with different spectral properties exist [199,200]). Recent work from the Jaffrey laboratory showed how Spinach can be adapted in a modular fashion into a combined sensor reporter of metabolites and proteins by altering a hairpin to contain a second aptamer [201]: binding of the target ligand allows the fluorophore binding pocket to form. Toehold switches can also be used to sense RNA levels [20], and *in vitro* studies that combine strand displacement sensing of RNA with the Spinach reporter show promise as future *in vivo* sensors that do not require an intermediate translation step [202,203]. Reporters and sensors can reveal the internal dynamics of gene circuits, but their introduction will perturb the gene circuit due to interactions with core components and the additional load on the host [193].

Tuneable elements in gene circuits

Once the cause of failure in a gene circuit has been identified, changes to the circuit must be made to correct its behaviour. Contextual interference might be resolved by changing the host chassis [44,176] or growth conditions. A component may have to be replaced due to toxic effects of its expression or because the required transfer function is outside of its dynamic range. If a component belongs to a large orthogonal set—for example, the TetR transcriptional regulators [21] or the IS10-based translational regulators [18]—then it may be possible to select an alternative that has a more appropriate transfer function. Signal amplifiers also can be introduced into the gene circuit to correct for impedance mismatch by modulating the output of an upstream component to harmonise the connection [45,162,204]. Otherwise, the behaviour of a gene circuit is typically corrected by making appropriate adjustments to promoter or RBS strength, component degradation rate, or copy number.

RBSs and promoters are well suited as tuning knobs for a gene circuit [44,205], as their (relative) activity can be computationally modelled reasonably well [31,117]; thus, the selection of variants is not limited to a characterised library. In addition, they are short sequences that have a large influence on the transfer function of a component, so a wide range of activities can be sampled by using degenerate oligonucleotides to construct or mutating these parts [115]. For example, the T7 promoter sequence is approximately 23 base pairs long, with distinct subsequences responsible for initiation and specificity [169] that have made it amenable to mutagenesis and screening for variants with a range of promoter strengths [206,207]. The interacting regions of *trans*- and *cis*-acting RNAs share the same qualities of predictable design and relative short length and thus could be useful as tuning devices.

The number of copies of a DNA sequence can be adjusted either through adding duplicates to the design of the circuit (e.g., doubling up transcriptional attenuators [85]) or by transferring the component to a plasmid

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

with a different copy number [208]. Control over the levels of DNA components can be used to tune transfer functions: increasing the copy number of a gene will increase the output mRNA level [208]; increasing the number of operator sites can improve cooperativity through regulator sequestration [209].

The degradation rate of components is an important parameter in dynamic circuits, such as repressilators, which require rapid degradation of regulators to remain in the oscillating parameter space [210]. Tools for controlling protein degradation rate were described above [132,134,136]; tools for controlling RNA degradation rates are less sophisticated, but we anticipate that the growth of RNA-based gene circuits will stimulate progress in this area.

Strategies for circuit refinement

When refining the behaviour of a gene circuit, the extent to which synthetic biologists should rely on rational design informed by characterisation, or directed evolution, is a matter of debate [211]. In practice, our current inability to accurately predict component function means that efficient tuning strategies will probably involve some screening of variants [7,212]. Whilst modern combinatorial assembly methods are powerful [40], there are limits to the size of variant libraries that can be effectively sampled in a screen [114]. Characterisation and subsequent modelling [44,107,196,213] of circuits can be used to inform the creation of variants, in order to efficiently search a circuit's parameter space for a desired behaviour [115]. Variation might also be included in the first iteration of circuit building, if potential points of failure can be identified in advance. The recent construction of a half-adder logic circuit in *E. coli* made use of both of these strategies, using the results from tests of lower-level circuits plus *a priori* knowledge of possible context-dependency-based "bugs", to create a focused selection of variant designs during the next stage of circuit development [214]. The requirement for tuning and debugging gene circuits is an important consideration when choosing an assembly strategy for construction of a gene circuit [215].

The widely used BioBrick standard [9,216,217] (and related variants [218,219]) uses type II restriction enzymes for the stepwise idempotent construction of units that have standard prefix and suffix linkers. Whilst the introduction of variation to tuning elements is easy during the initial assembly, once a part has been subsumed into a larger module, it cannot be exchanged; the workflow must return to the step where that part was added if alterations need to be made, or alternative (e.g., PCR-based) methods of introducing variation must be used. This cumbersome limitation is not found in the recently described BASIC method, which uses type IIS restriction enzymes to create overhangs for the attachment of linker fragments to defined parts, facilitating parallel assembly of up to seven components [220]. A similar method using isothermal assembly is available, but the linkers are defined when parts are initially cloned, again making it more difficult to change the order of parts [221].

Bespoke, PCR-based assembly methods such as SLIC [222], Gibson Assembly [223], CPEC [224], and SLiCE [225] are alternatives to linker-based assembly, and they avoid the formation of "scars" that can influence circuit function [226]. A large range of variation can easily be introduced to several positions in parallel using degenerate oligonucleotides [107], whereas modular assembly strategies might be limited by the characterised parts available. Construction is not always sequence independent, however, as secondary structure and repeated sequences in the overlaps can prevent correct annealing. Additionally, the requirement for generating parts by PCR (and subsequent sequence validation) for every construct may not be suitable for some workflows [220].

MAGE is a powerful tool for introducing post-assembly variation into a gene circuit [114,227]. ssDNA oligonucleotides are incorporated into replicating DNA via λ-Red recombination. Repeated rounds of MAGE with a mixture of oligonucleotides targeting different loci and/or with degenerate sequences [115] can be used to incrementally saturate the population with mutations.

Concluding Remarks and Outlook

After nearly one and a half decades of accelerating growth, synthetic biology has now advanced to a stage where there are many tools and building blocks available for gene circuit engineering. Significant progress has also been made to improve the modularity and orthogonality of these building blocks to enable more predictable and robust design of large complex gene networks. Using the state-of-the-art tools and principles described here, the scale, complexity, and resulting functionality of next-generation synthetic gene circuits will be significantly greater than their current counterparts, thus enabling many new applications and driving a revolution in industrial biotechnology.

RNA-based parts are likely to majorly drive the next increases in gene circuit sophistication due to their ease of programmability and because the thermodynamic models that facilitate their design can be adapted to predict behaviour at the circuit scale [228,229]. CRISPR-dCas9 and toehold switches are prime examples, exhibiting large dynamic ranges and specific interactions with targets. In addition, standardised expression cassettes will aid predictive design and tuning of protein components. The deployment of these tools in the near future should provide useful insights into their potential limitations (e.g., toxicity

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[26]) and best practice for their implementation. Likewise, detailed characterisation of the larger circuits that we anticipate being built in the coming years will inform our understanding of context effects and retroactivity, enabling engineers to hone design principles for improving robustness. Though the dynamic and undefined environment of the host can cause unexpected variation in gene circuit behaviour, strategies that combine the use of insulated parts and rational, model-driven searching of parameter space will reduce the time required to build large circuits. Variation due to interactions with the host metabolism might be further reduced by using strains with reduced genomes [192].

That said, further development of components is needed, especially in particular areas. For example, modular sensors of small molecules with new bespoke binding specificities will be required to connect synthetic control circuits with metabolic pathways [68]. Similarly, new orthogonal quorum-sensing mechanisms [230] would expand the opportunities for intercellular communication in synthetic microbial communities [231], enabling the development of more sophisticated distributed biological computing [232]. The ease of screening for aptamers puts the development of RNA-based sensors at the fore for now, though the potential for stronger interactions with a wider range of ligands means rationally engineered proteins will probably be better components in the future [233]. Work is needed to standardise and refine the process of linking RNA aptamers with regulatory regions, to produce components that possess high specificity and large dynamic ranges.

There are also opportunities to expand and improve existing classes of regulators: for example, the mechanism of STAR transcriptional regulation by direct disruption of a terminator hairpin [22] is very similar to the mechanism of toehold switch translational regulation [20], suggesting that the number of orthogonal STARs might be equally amenable to scale up through the rational design of new STAR-terminator pairs [173]. DNA-based memory will potentially enable biological circuits that can learn from past experience and adapt accordingly, but the slow rates of DNA modification make population-wide digital recording of transient signals difficult [25,164]. Two-tier memory systems may prove valuable, using bistable circuits with fast switching dynamics to stabilise a signal until complete DNA modification has occurred.

Recent advances in part provision and quality will need to be matched by development of circuit design capabilities if significant gains in complexity are to be realised. Computational tools for the selection and optimisation of network topology and constituent parts will be essential for the design of large synthetic networks [234–236]. Digital signal processing is required for certain applications [237] but is energetically expensive and therefore difficult to scale up [238]. A shift towards analogue signal processing will be required to create synthetic circuits that have the sophistication and efficiency of natural systems [11,238]. For the next generation of designer microbes to function robustly outside of the laboratory, circuit design will need to incorporate higher-level management of functional subsystems [239] to provide the adaptability necessary for real-world applications.

Acknowledgements

This work was supported by a Biotechnology and Biological Sciences Research Council project grant (BB/K016288/1) and a Royal Society grant award (RG120527). B.W. acknowledges support from the Scottish Universities Life Sciences Alliance and the Wellcome Trust Institutional Strategic Support Fund.

Received 12 June 2015;
Received in revised form 5 October 2015;
Accepted 6 October 2015
Available online xxxx

Keywords:
synthetic biology;
gene circuit;
engineering tools;
modularity and orthogonality;
transcriptional and translational control

Abbreviations used:
RBS, ribosome binding site; RNAP, RNA polymerase; sRNA, small RNA; UTR, untranslated region; UAA, unnatural amino acid; ssDNA, single-stranded DNA; taRNA, *trans*-acting RNA; GOI, gene of interest; TALE, transcription activator-like effector; CRISPR, clustered regularly interspaced short palindromic repeat; STAR, small transcription activating RNA; PAM, protospacer-adjacent motif; MAGE, multiplex automated genome engineering; EOU, expression operating unit.

References

[1] S. Mukherji, A. van Oudenaarden, Synthetic biology: Understanding biological design from synthetic circuits, Nat. Rev. Genet. 10 (2009) 859–871, http://dx.doi.org/10.1038/nrg2697.

[2] . D.-K. Ro, E.M. Paradise, M. Ouellet, K.J. Fisher, K.L. Newman, J.M. Ndungu, et al., Production of the antimalarial drug precursor artemisinic acid in engineered yeast, Nature 440 (2006) 940–943, http://dx.doi.org/10.1038/nature04640.

[3] B. Wang, M. Barahona, M. Buck, A modular cell-based biosensor using engineered genetic logic circuits to detect and integrate multiple environmental signals, Biosens. Bioelectron. 40 (2013) 368–376, http://dx.doi.org/10.1016/j.bios.2012.08.011.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[4] T. Danino, A. Prindle, G.A. Kwong, M. Skalak, H. Li, K. Allen, et al., Programmable probiotics for detection of cancer in urine, Sci. Transl. Med. 7 (2015) 289ra84, http://dx.doi.org/10.1126/scitranslmed.aaa3519.
[5] S. Gupta, E.E. Bram, R. Weiss, Genetically programmable pathogen sense and destroy, ACS Synth. Biol. 2 (2013) 715–723, http://dx.doi.org/10.1021/sb4000417.
[6] I.Y. Hwang, M.H. Tan, E. Koh, C.L. Ho, C.L. Poh, M.W. Chang, Reprogramming microbes to be pathogen-seeking killers, ACS Synth. Biol. 3 (2014) 228–237, http://dx.doi.org/10.1021/sb400077j.
[7] A.L. Slusarczyk, A. Lin, R. Weiss, Foundations for the design and implementation of synthetic genetic circuits, Nat. Rev. Genet. 13 (2012) 406–420, http://dx.doi.org/10.1038/nrg3227.
[8] A.A.K. Nielsen, T.H. Segall-Shapiro, C.A. Voigt, Advances in genetic circuit design: Novel biochemistries, deep part mining, and precision gene expression, Curr. Opin. Chem. Biol. 17 (2013) 878–892, http://dx.doi.org/10.1016/j.cbpa.2013.10.003.
[9] B. Canton, A. Labno, D. Endy, Refinement and standardization of synthetic biological parts and devices, Nat. Biotechnol. 26 (2008) 787–793, http://dx.doi.org/10.1038/nbt1413.
[10] C.A. Voigt, Genetic parts to program bacteria, Curr. Opin. Biotechnol. 17 (2006) 548–557, http://dx.doi.org/10.1016/j.copbio.2006.09.001.
[11] N. Roquet, T.K. Lu, Digital and analog gene circuits for biotechnology, Biotechnol. J. 9 (2014) 597–608, http://dx.doi.org/10.1002/biot.201300258.
[12] O. Purcell, T.K. Lu, Synthetic analog and digital circuits for cellular computation and memory, Curr. Opin. Biotechnol. 29 (2014) 146–155, http://dx.doi.org/10.1016/j.copbio.2014.04.009.
[13] B. Wang, M. Buck, Customizing cell signaling using engineered genetic logic circuits, Trends Microbiol. 20 (2012) 376–384, http://dx.doi.org/10.1016/j.tim.2012.05.001.
[14] S. Cardinale, A.P. Arkin, Contextualizing context for synthetic biology—Identifying causes of failure of synthetic biological systems, Biotechnol. J. 7 (2012) 856–866, http://dx.doi.org/10.1002/biot.201200085.
[15] A.P. Arkin, A wise consistency: Engineering biology for conformity, reliability, predictability, Curr. Opin. Chem. Biol. 17 (2013) 893–901, http://dx.doi.org/10.1016/j.cbpa.2013.09.012.
[16] L. Qi, R.E. Haurwitz, W. Shao, J.A. Doudna, A.P. Arkin, RNA processing enables predictable programming of gene expression, Nat. Biotechnol. 30 (2012) 1002–1006, http://dx.doi.org/10.1038/nbt.2355.
[17] V.K. Mutalik, J.C. Guimaraes, G. Cambray, C. Lam, M.J. Christoffersen, Q.-A. Mai, et al., Precise and reliable gene expression via standard transcription and translation initiation elements, Nat. Methods 10 (2013) 354–360, http://dx.doi.org/10.1038/nmeth.2404.
[18] V.K. Mutalik, L. Qi, J.C. Guimaraes, J.B. Lucks, A.P. Arkin, Rationally designed families of orthogonal RNA regulators of translation, Nat. Chem. Biol. 8 (2012) 447–454, http://dx.doi.org/10.1038/nchembio.919.
[19] L.S. Qi, M.H. Larson, L.A. Gilbert, J.A. Doudna, J.S. Weissman, A.P. Arkin, et al., Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression, Cell 152 (2013) 1173–1183, http://dx.doi.org/10.1016/j.cell.2013.02.022.
[20] A.A. Green, P.A. Silver, J.J. Collins, P. Yin, Toehold switches: De-novo-designed regulators of gene expression, Cell 159 (2014) 925–939, http://dx.doi.org/10.1016/j.cell.2014.10.002.
[21] B.C. Stanton, A.A.K. Nielsen, A. Tamsir, K. Clancy, T. Peterson, C.A. Voigt, Genomic mining of prokaryotic repressors for orthogonal logic gates, Nat. Chem. Biol. 10 (2013) 99–105, http://dx.doi.org/10.1038/nchembio.1411.
[22] J. Chappell, M.K. Takahashi, J.B. Lucks, Creating small transcription activating RNAs, Nat. Chem. Biol. 11 (2015) 1–9, http://dx.doi.org/10.1038/nchembio.1737.
[23] Y. Schaerli, M. Gili, M. Isalan, A split intein T7 RNA polymerase for transcriptional AND-logic, Nucleic Acids Res. 42 (2014) 12322–12328, http://dx.doi.org/10.1093/nar/gku884.
[24] J.E. Dueber, G.C. Wu, G.R. Malmirchegini, T.S. Moon, C.J. Petzold, A.V. Ullal, et al., Synthetic protein scaffolds provide modular control over metabolic flux, Nat. Biotechnol. 27 (2009) 753–759, http://dx.doi.org/10.1038/nbt.1557.
[25] F. Farzadfard, T.K. Lu, Genomically encoded analog memory with precise *in vivo* DNA writing in living cell populations, Science 346 (2014) 1256272, http://dx.doi.org/10.1126/science.1256272.
[26] J.A.N. Brophy, C.A. Voigt, Principles of genetic circuit design, Nat. Methods 11 (2014) 508–520, http://dx.doi.org/10.1038/nmeth.2926.
[27] L.S. Qi, A.P. Arkin, A versatile framework for microbial engineering using synthetic non-coding RNAs, Nat. Rev. Microbiol. 12 (2014) 341–354, http://dx.doi.org/10.1038/nrmicro3244.
[28] J. Mairhofer, A. Wittwer, M. Cserjan-Puschmann, G. Striedner, Preventing T7 RNA polymerase read-through transcription—A synthetic termination signal capable of improving bioprocess stability, ACS Synth. Biol. 4 (2015) 265–273, http://dx.doi.org/10.1021/sb5000115.
[29] P. Siuti, J. Yazbek, T.K. Lu, Synthetic circuits integrating logic and memory in living cells, Nat. Biotechnol. 31 (2013) 448–452, http://dx.doi.org/10.1038/nbt.2510.
[30] C. Lou, B. Stanton, Y.-J. Chen, B. Munsky, C.A. Voigt, Ribozyme-based insulator parts buffer synthetic circuits from genetic context, Nat. Biotechnol. 22 (2012) 349–355, http://dx.doi.org/10.1038/nbt.2401.
[31] R.C. Brewster, D.L. Jones, R. Phillips, Tuning promoter strength through RNA polymerase binding site design in *Escherichia coli*, PLoS Comput. Biol. 8 (2012) e1002811, http://dx.doi.org/10.1371/journal.pcbi.1002811.
[32] V.A. Rhodius, T.H. Segall-Shapiro, B.D. Sharon, A. Ghodasara, E. Orlova, H. Tabakh, et al., Design of orthogonal genetic switches based on a crosstalk map of σs, anti-σs, and promoters, Mol. Syst. Biol. 9 (2013) 702, http://dx.doi.org/10.1038/msb.2013.58.
[33] D. Bikard, W. Jiang, P. Samai, A. Hochschild, F. Zhang, L.A. Marraffini, Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system, Nucleic Acids Res. 41 (2013) 7429–7437, http://dx.doi.org/10.1093/nar/gkt520.
[34] S.T. Estrem, T. Gaal, W. Ross, R.L. Gourse, Identification of an UP element consensus sequence for bacterial promoters, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 9761–9766, http://dx.doi.org/10.1073/pnas.95.17.9761.
[35] M. De Mey, J. Maertens, G.J. Lequeux, W.K. Soetaert, E.J. Vandamme, Construction and model-based analysis of a promoter library for *E. coli*: An indispensable tool for metabolic engineering, BMC Biotechnol. 7 (2007) 34, http://dx.doi.org/10.1186/1472-6750-7-34.
[36] P.R. Jensen, K. Hammer, The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters, Appl. Environ. Microbiol. 64 (1998) 82–87.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[37] V.A. Rhodius, V.K. Mutalik, C.A. Gross, Predicting the strength of UP-elements and full-length *E. coli* sigma-E promoters, Nucleic Acids Res. 40 (2012) 2907–2924, http://dx.doi.org/10.1093/nar/gkr1190.

[38] J.H. Davis, A.J. Rubin, R.T. Sauer, Design, construction and characterization of a set of insulated bacterial promoters, Nucleic Acids Res. 39 (2011) 1131–1141, http://dx.doi.org/10.1093/nar/gkq810.

[39] J.R. Kelly, A.J. Rubin, J.H. Davis, C.M. Ajo-Franklin, J. Cumbers, M.J. Czar, et al., Measuring the activity of BioBrick promoters using an *in vivo* reference standard, J. Biol. Eng. 3 (2009) 4, http://dx.doi.org/10.1186/1754-1611-3-4.

[40] S. Kosuri, D.B. Goodman, G. Cambray, V.K. Mutalik, Y. Gao, A.P. Arkin, et al., Composability of regulatory sequences controlling transcription and translation in *Escherichia coli*, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 14024–14029, http://dx.doi.org/10.1073/pnas.1301301110.

[41] A.M. Stevens, N. Fujita, A. Ishihama, E.P. Greenberg, Involvement of the RNA polymerase alpha-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes, J. Bacteriol. 181 (1999) 4704–4707.

[42] T.S. Moon, C. Lou, A. Tamsir, B.C. Stanton, C.A. Voigt, Genetic programs constructed from layered logic gates in single cells, Nature 491 (2012) 249–253, http://dx.doi.org/10.1038/nature11516.

[43] N. Quiles-Puchalt, M.Á. Tormo-Más, S. Campoy, A. Toledo-Arana, V. Monedero, I. Lasa, et al., A super-family of transcriptional activators regulates bacteriophage packaging and lysis in Gram-positive bacteria, Nucleic Acids Res. 41 (2013) 7260–7275, http://dx.doi.org/10.1093/nar/gkt508.

[44] B. Wang, R.I. Kitney, N. Joly, M. Buck, Engineering modular and orthogonal genetic logic gates for robust digital-like synthetic biology, Nat. Commun. 2 (2011) 508, http://dx.doi.org/10.1038/ncomms1516.

[45] B. Wang, M. Barahona, M. Buck, Engineering modular and tunable genetic amplifiers for scaling transcriptional signals in cascaded gene networks, Nucleic Acids Res. 42 (2014) 9484–9492, http://dx.doi.org/10.1093/nar/gku593.

[46] M. Jovanovic, E.H. James, P.C. Burrows, F.G.M. Rego, M. Buck, J. Schumacher, Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity, Nat. Commun. 2 (2011) 177, http://dx.doi.org/10.1038/ncomms1177.

[47] E. Lawton, M. Jovanovic, N. Joly, C. Waite, N. Zhang, B. Wang, et al., Determination of the self-association residues within a homomeric and a heteromeric AAA+ enhancer binding protein, J. Mol. Biol. 426 (2014) 1692–1710, http://dx.doi.org/10.1016/j.jmb.2014.01.001.

[48] B. Wang, R. Kitney, M. Buck, M. Jovanovic, N. Joly, E. James, The design and construction of a set of modular synthetic BioLogic devices for programming cells, IFMBE Proc. 25/8 (2009) 289–292, http://dx.doi.org/10.1007/978-3-642-03887-7.

[49] K. Temme, R. Hill, T.H. Segall-Shapiro, F. Moser, C.A. Voigt, Modular control of multiple pathways using engineered orthogonal T7 polymerases, Nucleic Acids Res. 40 (2012) 8773–8781, http://dx.doi.org/10.1093/nar/gks597.

[50] A.J. Meyer, J.W. Ellefson, A.D. Ellington, Directed evolution of a panel of orthogonal T7 RNA polymerase variants for *in vivo* or *in vitro* synthetic circuitry, ACS Synth. Biol. (2014), http://dx.doi.org/10.1021/sb500299c.

[51] D.L. Shis, M.R. Bennett, Library of synthetic transcriptional AND gates built with split T7 RNA polymerase mutants, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 5028–5033, http://dx.doi.org/10.1073/pnas.1220157110.

[52] T.H. Segall-Shapiro, A.J. Meyer, A.D. Ellington, E.D. Sontag, C.A. Voigt, A "resource allocator" for transcription based on a highly fragmented T7 RNA polymerase, Mol. Syst. Biol. 10 (2014) 742, http://dx.doi.org/10.15252/msb.20145299.

[53] S. Itzkovitz, T. Tlusty, U. Alon, Coding limits on the number of transcription factors, BMC Genomics 7 (2006) 239, http://dx.doi.org/10.1186/1471-2164-7-239.

[54] J. Zhan, B. Ding, R. Ma, X. Ma, X. Su, Y. Zhao, et al., Develop reusable and combinable designs for transcriptional logic gates, Mol. Syst. Biol. 6 (2010) 388, http://dx.doi.org/10.1038/msb.2010.42.

[55] M.F. Copeland, M.C. Politz, B.F. Pfleger, Application of TALEs, CRISPR/Cas and sRNAs as *trans*-acting regulators in prokaryotes, Curr. Opin. Biotechnol. 29 (2014) 46–54, http://dx.doi.org/10.1016/j.copbio.2014.02.010.

[56] R. Moore, A. Chandrahas, L. Bleris, Transcription activator-like effectors: A toolkit for synthetic biology, ACS Synth. Biol. (2014), http://dx.doi.org/10.1021/sb400137b.

[57] J.Y. Lee, B.H. Sung, B.J. Yu, J.H. Lee, S.H. Lee, M.S. Kim, et al., Phenotypic engineering by reprogramming gene transcription using novel artificial transcription factors in *Escherichia coli*, Nucleic Acids Res. (2008), http://dx.doi.org/10.1093/nar/gkn449.

[58] A.C. Mercer, T. Gaj, R.P. Fuller, C.F. Barbas, Chimeric TALE recombinases with programmable DNA sequence specificity, Nucleic Acids Res. 40 (2012) 11163–11172, http://dx.doi.org/10.1093/nar/gks875.

[59] A. Garg, J.J. Lohmueller, P.A. Silver, T.Z. Armel, Engineering synthetic TAL effectors with orthogonal target sites, Nucleic Acids Res. 40 (2012) 7584–7595, http://dx.doi.org/10.1093/nar/gks404.

[60] M.C. Politz, M.F. Copeland, B.F. Pfleger, Artificial repressors for controlling gene expression in bacteria, Chem. Commun. 49 (2013) 4325–4327, http://dx.doi.org/10.1039/c2cc37107c.

[61] J. Liang, R. Chao, Z. Abil, Z. Bao, H. Zhao, FairyTALE: A high-throughput TAL effector synthesis platform, ACS Synth. Biol. (2013), http://dx.doi.org/10.1021/sb400109p.

[62] J.L. Schmid-Burgk, T. Schmidt, V. Kaiser, K. Höning, V. Hornung, A ligation-independent cloning technique for high-throughput assembly of transcription activator-like effector genes, Nat. Biotechnol. 31 (2013) 76–81, http://dx.doi.org/10.1038/nbt.2460.

[63] A.W. Briggs, X. Rios, R. Chari, L. Yang, F. Zhang, P. Mali, et al., Iterative capped assembly: Rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers, Nucleic Acids Res. 40 (2012) 1–10, http://dx.doi.org/10.1093/nar/gks624.

[64] A. Tamsir, J.J. Tabor, C.A. Voigt, Robust multicellular computing using genetically encoded NOR gates and chemical "wires", Nature 469 (2011) 212–215, http://dx.doi.org/10.1038/nature09565.

[65] T.A. Desai, D.A. Rodionov, M.S. Gelfand, E.J. Alm, C.V. Rao, Engineering transcription factors with novel DNA-binding specificity using comparative genomics, Nucleic Acids Res. 37 (2009) 2493–2503, http://dx.doi.org/10.1093/nar/gkp079.

[66] B. Wang, M. Barahona, M. Buck, J. Schumacher, Rewiring cell signalling through chimaeric regulatory protein engineering, Biochem. Soc. Trans. 41 (2013) 1195–1200, http://dx.doi.org/10.1042/BST20130138.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[67] E.L.C. De los Santos, J.T. Meyerowitz, S.L. Mayo, R.M. Murray, Engineering transcriptional regulator effector specificity using computational design and *in vitro* rapid prototyping: Developing a vanillin, Sensors (2015), http://dx.doi.org/10.1101/015438.
[68] R.H. Dahl, F. Zhang, J. Alonso-Gutierrez, E. Baidoo, T.S. Batth, A.M. Redding-Johanson, et al., Engineering dynamic pathway regulation using stress-response promoters, Nat. Biotechnol. 31 (2013) 1039–1046, http://dx.doi.org/10.1038/nbt.2689.
[69] C. Berens, F. Groher, B. Suess, RNA aptamers as genetic control devices: The potential of riboswitches as synthetic elements for regulating gene expression, Biotechnol. J. 10 (2015) 246–257, http://dx.doi.org/10.1002/biot.201300498.
[70] M. Wachsmuth, S. Findeiß, N. Weissheimer, P.F. Stadler, M. Mörl, *De novo* design of a synthetic riboswitch that regulates transcription termination, Nucleic Acids Res. 41 (2013) 2541–2551, http://dx.doi.org/10.1093/nar/gks1330.
[71] P. Ceres, A.D. Garst, J.G. Marcano-Velázquez, R.T. Batey, Modularity of select riboswitch expression platforms enables facile engineering of novel genetic regulatory devices, ACS Synth. Biol. 2 (2013) 463–472, http://dx.doi.org/10.1021/sb4000096.
[72] P. Ceres, J.J. Trausch, R.T. Batey, Engineering modular "ON" RNA switches using biological components, Nucleic Acids Res. 41 (2013) 10449–10461, http://dx.doi.org/10.1093/nar/gkt787.
[73] N. Dixon, J.N. Duncan, T. Geerlings, M.S. Dunstan, J.E.G. McCarthy, D. Leys, et al., Reengineering orthogonally selective riboswitches, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2830–2835, http://dx.doi.org/10.1073/pnas.0911209107.
[74] C.J. Robinson, H.A. Vincent, M.C. Wu, P.T. Lowe, M.S. Dunstan, D. Leys, et al., Modular riboswitch toolsets for synthetic genetic control in diverse bacterial species, J. Am. Chem. Soc. 136 (2014) 10615–10624, http://dx.doi.org/10.1021/ja502873j.
[75] A.D. Ellington, J.W. Szostak, *In vitro* selection of RNA molecules that bind specific ligands, Nature 346 (1990) 818–822, http://dx.doi.org/10.1038/346818a0.
[76] S.A. Lynch, J.P. Gallivan, A flow cytometry-based screen for synthetic riboswitches, Nucleic Acids Res. 37 (2009) 184–192, http://dx.doi.org/10.1093/nar/gkn924.
[77] C. Berens, B. Suess, Riboswitch engineering—Making the all-important second and third steps, Curr. Opin. Biotechnol. 31 (2015) 10–15, http://dx.doi.org/10.1016/j.copbio.2014.07.014.
[78] C.C. Liu, L. Qi, C. Yanofsky, A.P. Arkin, Regulation of transcription by unnatural amino acids, Nat. Biotechnol. 29 (2011) 164–168, http://dx.doi.org/10.1038/nbt.1741.
[79] A. Levskaya, A.A. Chevalier, J.J. Tabor, Z.B. Simpson, L.A. Lavery, M. Levy, et al., Synthetic biology: Engineering *Escherichia coli* to see light, Nature 438 (2005) 441–442, http://dx.doi.org/10.1038/nature04405.
[80] J.J. Tabor, H.M. Salis, Z.B. Simpson, A.A. Chevalier, A. Levskaya, E.M. Marcotte, et al., A synthetic genetic edge detection program, Cell 137 (2009) 1272–1281, http://dx.doi.org/10.1016/j.cell.2009.04.048.
[81] J.J. Tabor, A. Levskaya, C.A. Voigt, Multichromatic control of gene expression in *Escherichia coli*, J. Mol. Biol. 405 (2011) 315–324, http://dx.doi.org/10.1016/j.jmb.2010.10.038.
[82] R. Ohlendorf, R.R. Vidavski, A. Eldar, K. Moffat, A. Möglich, From dusk till dawn: One-plasmid systems for light-regulated gene expression, J. Mol. Biol. 416 (2012) 534–542, http://dx.doi.org/10.1016/j.jmb.2012.01.001.
[83] G. Rodrigo, T.E. Landrain, S. Shen, A. Jaramillo, A new frontier in synthetic biology: Automated design of small RNA devices in bacteria, Trends Genet. 29 (2013) 529–536, http://dx.doi.org/10.1016/j.tig.2013.06.005.
[84] J. Chappell, M.K. Takahashi, S. Meyer, D. Loughrey, K.E. Watters, J. Lucks, The centrality of RNA for engineering gene expression, Biotechnol. J. 8 (2013) 1379–1395, http://dx.doi.org/10.1002/biot.201300018.
[85] J.B. Lucks, L. Qi, V.K. Mutalik, D. Wang, A.P. Arkin, Versatile RNA-sensing transcriptional regulators for engineering genetic networks, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 8617–8622, http://dx.doi.org/10.1073/pnas.1015741108.
[86] M.K. Takahashi, J.B. Lucks, A modular strategy for engineering orthogonal chimeric RNA transcription regulators, Nucleic Acids Res. 41 (2013) 7577–7588, http://dx.doi.org/10.1093/nar/gkt452.
[87] L. Qi, J.B. Lucks, C.C. Liu, V.K. Mutalik, A.P. Arkin, Engineering naturally occurring *trans*-acting non-coding RNAs to sense molecular signals, Nucleic Acids Res. 40 (2012) 5775–5786, http://dx.doi.org/10.1093/nar/gks168.
[88] C.C. Liu, L. Qi, J.B. Lucks, T.H. Segall-Shapiro, D. Wang, V.K. Mutalik, et al., An adaptor from translational to transcriptional control enables predictable assembly of complex regulation, Nat. Methods 9 (2012) 1088–1094, http://dx.doi.org/10.1038/nmeth.2184.
[89] P.C. Fineran, R.L. Dy, Gene regulation by engineered CRISPR-Cas systems, Curr. Opin. Microbiol. 18 (2014) 83–89, http://dx.doi.org/10.1016/j.mib.2014.02.007.
[90] Y. Jiang, B. Chen, C. Duan, B. Sun, J. Yang, S. Yang, Multigene editing in the *Escherichia coli* genome using the CRISPR-Cas9 system, Appl. Environ. Microbiol. (2015), http://dx.doi.org/10.1128/AEM.04023-14.
[91] R.E. Cobb, Y. Wang, H. Zhao, High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system, ACS Synth. Biol. (2014), http://dx.doi.org/10.1021/sb500351f.
[92] J.E. Dicarlo, J.E. Norville, P. Mali, X. Rios, J. Aach, G.M. Church, Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems, Nucleic Acids Res. 41 (2013) 4336–4343, http://dx.doi.org/10.1093/nar/gkt135.
[93] W. Jiang, D. Bikard, D. Cox, F. Zhang, L.A. Marraffini, RNA-guided editing of bacterial genomes using CRISPR-Cas systems, Nat. Biotechnol. 31 (2013) 233–239, http://dx.doi.org/10.1038/nbt.2508.
[94] F.A. Ran, P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, F. Zhang, Genome engineering using the CRISPR-Cas9 system, Nat. Protoc. 8 (2013) 2281–2308, http://dx.doi.org/10.1038/nprot.2013.143.
[95] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821, http://dx.doi.org/10.1126/science.1225829.
[96] S.H. Sternberg, S. Redding, M. Jinek, E.C. Greene, J.A. Doudna, DNA interrogation by the CRISPR RNA-guided endonuclease Cas9, Nature 507 (2014) 62–67, http://dx.doi.org/10.1038/nature13011.
[97] B. Zetsche, S.E. Volz, F. Zhang, A split-Cas9 architecture for inducible genome editing and transcription modulation, Nat. Biotechnol. 33 (2015) 139–142, http://dx.doi.org/10.1038/nbt.3149.
[98] A.V. Wright, S.H. Sternberg, D.W. Taylor, B.T. Staahl, J.A. Bardales, J.E. Kornfeld, et al., Rational design of a split-Cas9 enzyme complex, Proc. Natl. Acad. Sci. 112 (2015) 201501698, http://dx.doi.org/10.1073/pnas.1501698112.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[99] B.F. Cress, Ö.D. Toparlak, S. Guleria, M. Lebovich, J.T. Stieglitz, J.A. Englaender, et al., CRISPathBrick: Modular combinatorial assembly of type II-A CRISPR arrays for dCas9-mediated multiplex transcriptional repression in *E. coli*, ACS Synth. Biol. (2015), http://dx.doi.org/10.1021/acssynbio.5b00012.

[100] A.A. Nielsen, C.A. Voigt, Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks, Mol. Syst. Biol. (2014), http://dx.doi.org/10.15252/msb.20145735.

[101] J.G. Zalatan, M.E. Lee, R. Almeida, L.A. Gilbert, E.H. Whitehead, M. La Russa, et al., Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds, Cell 160 (2014) 339–350, http://dx.doi.org/10.1016/j.cell.2014.11.052.

[102] M.R. O'Connell, B.L. Oakes, S.H. Sternberg, A. East-Seletsky, M. Kaplan, J.A. Doudna, Programmable RNA recognition and cleavage by CRISPR/Cas9, Nature 516 (2014) 263–266, http://dx.doi.org/10.1038/nature13769.

[103] D.A. Nelles, M.Y. Fang, S. Aigner, G.W. Yeo, Applications of Cas9 as an RNA-programmed RNA-binding protein, BioEssays 37 (2015) 732–739, http://dx.doi.org/10.1002/bies.201500001.

[104] G. Tamulaitis, M. Kazlauskiene, E. Manakova, Č. Venclovas, A.O. Nwokeoji, M.J. Dickman, et al., Programmable RNA shredding by the type III-A CRISPR-Cas system of *Streptococcus thermophilus*, Mol. Cell 56 (2014) 506–517, http://dx.doi.org/10.1016/j.molcel.2014.09.027.

[105] M.N. Win, C.D. Smolke, A modular and extensible RNA-based gene-regulatory platform for engineering cellular function, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 14283–14288, http://dx.doi.org/10.1073/pnas.0703961104.

[106] N. Nakashima, T. Tamura, Gene silencing in *Escherichia coli* using antisense RNAs expressed from doxycycline-inducible vectors, Lett. Appl. Microbiol. 56 (2013) 436–442, http://dx.doi.org/10.1111/lam.12066.

[107] I. Farasat, M. Kushwaha, J. Collens, M. Easterbrook, M. Guido, H.M. Salis, Efficient search, mapping, and optimization of multi-protein genetic systems in diverse bacteria, Mol. Syst. Biol. 10 (2014) 731, http://dx.doi.org/10.15252/msb.20134955.

[108] T.A. Carrier, J.D. Keasling, Library of synthetic 5′ secondary structures to manipulate mRNA stability in *Escherichia coli*, Biotechnol. Prog. 15 (1999) 58–64, http://dx.doi.org/10.1021/bp9801143.

[109] B. Reeve, T. Hargest, C. Gilbert, T. Ellis, Predicting translation initiation rates for designing synthetic biology, Front. Bioeng. Biotechnol. 2 (2014) 1–6, http://dx.doi.org/10.3389/fbioe.2014.00001.

[110] H.M. Salis, E.A. Mirsky, C.A. Voigt, Automated design of synthetic ribosome binding sites to control protein expression, Nat. Biotechnol. 27 (2009) 946–950, http://dx.doi.org/10.1038/nbt.1568.

[111] D. Na, D. Lee, RBSDesigner: Software for designing synthetic ribosome binding sites that yield a desired level of protein expression, Bioinformatics 3 (2010) 1–2, http://dx.doi.org/10.1093/bioinformatics/btq458.

[112] S.W. Seo, J.S. Yang, I. Kim, J. Yang, B.E. Min, S. Kim, et al., Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency, Metab. Eng. 15 (2013) 67–74, http://dx.doi.org/10.1016/j.ymben.2012.10.006.

[113] S.W. Seo, J.-S. Yang, H.-S. Cho, J. Yang, S.C. Kim, J.M. Park, et al., Predictive combinatorial design of mRNA translation initiation regions for systematic optimization of gene expression levels, Sci. Rep. 4 (2014) 4515, http://dx.doi.org/10.1038/srep04515.

[114] R.R. Gallagher, Z. Li, A.O. Lewis, F.J. Isaacs, Rapid editing and evolution of bacterial genomes using libraries of synthetic DNA, Nat. Protoc. 9 (2014) 2301–2316, http://dx.doi.org/10.1038/nprot.2014.082.

[115] C.Y. Ng, I. Farasat, C.D. Maranas, H.M. Salis, Rational design of a synthetic Entner-Doudoroff pathway for improved and controllable NADPH regeneration, Metab. Eng. 29 (2015) 86–96, http://dx.doi.org/10.1016/j.ymben.2015.03.001.

[116] H.M. Salis, The ribosome binding site calculator, Methods Enzymol. 498 (2011) 19–42, http://dx.doi.org/10.1016/B978-0-12-385120-8.00002-4.

[117] A. Espah Borujeni, A.S. Channarasappa, H.M. Salis, Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites, Nucleic Acids Res. 42 (2014) 2646–2659, http://dx.doi.org/10.1093/nar/gkt1139.

[118] O. Rackham, J.W. Chin, A network of orthogonal ribosome x mRNA pairs, Nat. Chem. Biol. 1 (2005) 159–166, http://dx.doi.org/10.1038/nchembio719.

[119] W. An, J.W. Chin, Synthesis of orthogonal transcription-translation networks, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 8477–8482, http://dx.doi.org/10.1073/pnas.0900267106.

[120] G.-W. Li, E. Oh, J.S. Weissman, The anti-Shine–Dalgarno sequence drives translational pausing and codon choice in bacteria, Nature 484 (2012) 538–541, http://dx.doi.org/10.1038/nature10965.

[121] S. Topp, J.P. Gallivan, Guiding bacteria with small molecules and RNA, J. Am. Chem. Soc. 129 (2008) 6807–6811, http://dx.doi.org/10.1021/ja0692480.Guiding.

[122] V. Sharma, Y. Nomura, Y. Yokobayashi, Engineering complex riboswitch regulation by dual genetic selection, J. Am. Chem. Soc. 130 (2008) 16310–16315, http://dx.doi.org/10.1021/ja805203w.

[123] S.S. Krajewski, F. Narberhaus, Temperature-driven differential gene expression by RNA thermosensors, Biochim. Biophys. Acta, Gene Regul. Mech. (2014), http://dx.doi.org/10.1016/j.bbagrm.2014.03.006.

[124] D. Na, S.M. Yoo, H. Chung, H. Park, J.H. Park, S.Y. Lee, Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs, Nat. Biotechnol. 31 (2013) 170–174, http://dx.doi.org/10.1038/nbt.2461.

[125] H. Aiba, Mechanism of RNA silencing by Hfq-binding small RNAs, Curr. Opin. Microbiol. 10 (2007) 134–139, http://dx.doi.org/10.1016/j.mib.2007.03.010.

[126] V. Sharma, A. Yamamura, Y. Yokobayashi, Engineering artificial small RNAs for conditional gene silencing in *Escherichia coli*, ACS Synth. Biol. 1 (2012) 6–13, http://dx.doi.org/10.1021/sb200001q.

[127] F.J. Isaacs, D.J. Dwyer, C. Ding, D.D. Pervouchine, C.R. Cantor, J.J. Collins, Engineered riboregulators enable post-transcriptional control of gene expression, Nat. Biotechnol. 22 (2004) 841–847, http://dx.doi.org/10.1038/nbt986.

[128] J.M. Callura, C.R. Cantor, J.J. Collins, Genetic switchboard for synthetic biology applications, Proc. Natl. Acad. Sci. 109 (2012) 5850–5855, http://dx.doi.org/10.1073/pnas.1203808109.

[129] M. Krishnamurthy, S.P. Hennelly, T. Dale, S.R. Starkenburg, R. Marti-Arbona, D.T. Fox, et al., Tunable riboregulator switches for post-transcriptional control of gene expression, ACS Synth. Biol. (2015), http://dx.doi.org/10.1021/acssynbio.5b00041.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[130] C. Myhrvold, P.A. Silver, Using synthetic RNAs as scaffolds and regulators, Nat. Struct. Mol. Biol. 22 (2015) 8–10, http://dx.doi.org/10.1038/nsmb.2944.
[131] K. Pardee, A.A. Green, T. Ferrante, D.E. Cameron, A. DaleyKeyser, P. Yin, et al., Paper-based synthetic gene networks, Cell 1–15 (2014), http://dx.doi.org/10.1016/j.cell.2014.10.004.
[132] K.E. McGinness, T.A. Baker, R.T. Sauer, Engineering controllable protein degradation, Mol. Cell 22 (2006) 701–707, http://dx.doi.org/10.1016/j.molcel.2006.04.027.
[133] A. Prindle, J. Selimkhanov, H. Li, I. Razinkov, L.S. Tsimring, J. Hasty, Rapid and tunable post-translational coupling of genetic circuits, Nature 508 (2014) 387–391, http://dx.doi.org/10.1038/nature13238.
[134] J.H. Davis, T.A. Baker, R.T. Sauer, Small-molecule control of protein degradation using split adaptors, ACS Chem. Biol. 6 (2011) 1205–1213, http://dx.doi.org/10.1021/cb2001389.
[135] I.M. Brockman, K.L.J. Prather, Dynamic knockdown of *E. coli* central metabolism for redirecting fluxes of primary metabolites, Metab. Eng. 28 (2015) 104–113, http://dx.doi.org/10.1016/j.ymben.2014.12.005.
[136] D.E. Cameron, J.J. Collins, Tunable protein degradation in bacteria, Nat. Biotechnol. (2014), http://dx.doi.org/10.1038/nbt.3053.
[137] K.S. Nilgiriwala, J. Jiménez, P.M. Rivera, D. Del Vecchio, Synthetic tunable amplifying buffer circuit in *E. coli*, ACS Synth. Biol. 4 (2015) 577–584, http://dx.doi.org/10.1021/sb5002533.
[138] R. Grünberg, L. Serrano, Strategies for protein synthetic biology, Nucleic Acids Res. 38 (2010) 2663–2675, http://dx.doi.org/10.1093/nar/gkq139.
[139] B. Wang, M. Buck, Rapid engineering of versatile molecular logic gates using heterologous genetic transcriptional modules, Chem. Commun. (Camb.) 50 (2014) 11642–11644, http://dx.doi.org/10.1039/c4cc05264a.
[140] N.E. Buchler, F.R. Cross, Protein sequestration generates a flexible ultrasensitive response in a genetic network, Mol. Syst. Biol. 5 (2009) 272, http://dx.doi.org/10.1038/msb.2009.30.
[141] T.-H. Lee, N. Maheshri, A regulatory role for repeated decoy transcription factor binding sites in target gene expression, Mol. Syst. Biol. 8 (2012) 1–11, http://dx.doi.org/10.1038/msb.2012.7.
[142] S. Legewie, D. Dienst, A. Wilde, H. Herzel, I.M. Axmann, Small RNAs establish delays and temporal thresholds in gene expression, Biophys. J. 95 (2008) 3232–3238, http://dx.doi.org/10.1529/biophysj.108.133819.
[143] Q. Zhang, S. Bhattacharya, M.E. Andersen, Ultrasensitive response motifs: Basic amplifiers in molecular signalling networks, Open Biol. 3 (2013) 130031, http://dx.doi.org/10.1098/rsob.130031.
[144] W. Sun, J. Yang, X.-Q. Liu, Synthetic two-piece and three-piece split inteins for protein *trans*-splicing, J. Biol. Chem. 279 (2004) 35281–35286, http://dx.doi.org/10.1074/jbc.M405491200.
[145] E.R. Lee, J.L. Baker, Z. Weinberg, N. Sudarsan, R.R. Breaker, An allosteric self-splicing ribozyme triggered by a bacterial second messenger, Science (New York, N.Y.) 329 (2010) 845–848, http://dx.doi.org/10.1126/science.1190713.
[146] G. Hausner, M. Hafez, D.R. Edgell, Bacterial group I introns: Mobile RNA catalysts, Mob. DNA 5 (2014) 8, http://dx.doi.org/10.1186/1759-8753-5-8.
[147] S.A. Woodson, Ironing out the kinks: Splicing and translation in bacteria, Genes Dev. 12 (1998) 1243–1247, http://dx.doi.org/10.1101/gad.12.9.1243.
[148] R.A. Pitner, A.H. Scarpelli, J.N. Leonard, Regulation of bacterial gene expression by Protease-Alleviated Spatial Sequestration (PASS), ACS Synth. Biol. (2015), http://dx.doi.org/10.1021/sb500302y.
[149] S.-H. Park, A. Zarrinpar, W.A. Lim, Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms, Science (New York, N.Y.) 299 (2003) 1061–1064, http://dx.doi.org/10.1126/science.1076979.
[150] V. Hsiao, E.L.C. de Los Santos, W.R. Whitaker, J.E. Dueber, R.M. Murray, Design and implementation of a biomolecular concentration tracker, ACS Synth. Biol. 4 (2014) 150–161, http://dx.doi.org/10.1021/sb500024b.
[151] W.F. Rurup, J. Snijder, M.S.T. Koay, A.J.R. Heck, J.J.L.M. Cornelissen, Self-sorting of foreign proteins in a bacterial nanocompartment, J. Am. Chem. Soc. 136 (2014) 3828–3832, http://dx.doi.org/10.1021/ja410891c.
[152] A.D. Lawrence, S. Frank, S. Newnham, M.J. Lee, I.R. Brown, W. Xue, et al., Solution structure of a bacterial microcompartment targeting peptide and its application in the construction of an ethanol bioreactor, ACS Synth. Biol. 3 (2014) 454–465, http://dx.doi.org/10.1021/sb4001118.
[153] H. Lee, W.C. DeLoache, J.E. Dueber, Spatial organization of enzymes for metabolic engineering, Metab. Eng. 14 (2012) 242–251, http://dx.doi.org/10.1016/j.ymben.2011.09.003.
[154] T.S. Moon, J.E. Dueber, E. Shiue, K.L.J. Prather, Use of modular, synthetic scaffolds for improved production of glucaric acid in engineered *E. coli*, Metab. Eng. 12 (2010) 298–305, http://dx.doi.org/10.1016/j.ymben.2010.01.003.
[155] C.J. Delebecque, A.B. Lindner, P.A. Silver, F.A. Aldaye, Organization of intracellular reactions with rationally designed RNA assemblies, Science (New York, N.Y.) 333 (2011) 470–474, http://dx.doi.org/10.1126/science.1206938.
[156] W. Bacchus, M. Fussenegger, The use of light for engineered control and reprogramming of cellular functions, Curr. Opin. Biotechnol. 23 (2012) 695–702, http://dx.doi.org/10.1016/j.copbio.2011.12.004.
[157] H. Liu, X. Yu, K. Li, J. Klejnot, H. Yang, D. Lisiero, et al., Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in *Arabidopsis*, Science (New York, N.Y.) 322 (2008) 1535–1539, http://dx.doi.org/10.1126/science.1163927.
[158] M. Ni, J.M. Tepperman, P.H. Quail, Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light, Nature 400 (1999) 781–784, http://dx.doi.org/10.1038/23500.
[159] J.W. Kotula, S.J. Kerns, L.A. Shaket, L. Siraj, J.J. Collins, J.C. Way, et al., Programmable bacteria detect and record an environmental signal in the mammalian gut, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 4838–4843, http://dx.doi.org/10.1073/pnas.1321321111.
[160] T.S. Gardner, C.R. Cantor, J.J. Collins, Construction of a genetic toggle switch in *Escherichia coli*, Nature 403 (2000) 339–342, http://dx.doi.org/10.1038/35002131.
[161] D. Chen, A.P. Arkin, Sequestration-based bistability enables tuning of the switching boundaries and design of a latch, Mol. Syst. Biol. 8 (2012) 1–7, http://dx.doi.org/10.1038/msb.2012.52.
[162] J. Bonnet, P. Yin, M.E. Ortiz, P. Subsoontorn, D. Endy, Amplifying genetic logic gates, Science (New York, N.Y.) 340 (2013) 599–603, http://dx.doi.org/10.1126/science.1232758.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[163] L. Yang, A.A.K. Nielsen, J. Fernandez-Rodriguez, C.J. McClune, M.T. Laub, T.K. Lu, et al., Permanent genetic memory with >1-byte capacity, Nat. Methods (2014), http://dx.doi.org/10.1038/nmeth.3147.
[164] J. Bonnet, P. Subsoontorn, D. Endy, Rewritable digital data storage in live cells via engineered control of recombination directionality, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 8884–8889, http://dx.doi.org/10.1073/pnas.1202344109.
[165] D. Del Vecchio, A.J. Ninfa, E.D. Sontag, Modular cell biology: Retroactivity and insulation, Mol. Syst. Biol. 4 (2008) 161, http://dx.doi.org/10.1038/msb4100204.
[166] V.K. Mutalik, J.C. Guimaraes, G. Cambray, Q.-A. Mai, M.J. Christoffersen, L. Martin, et al., Quantitative estimation of activity and quality for collections of functional genetic elements, Nat. Methods 10 (2013) 347–353, http://dx.doi.org/10.1038/nmeth.2403.
[167] S. Jayanthi, K.S. Nilgiriwala, D. Del Vecchio, Retroactivity controls the temporal dynamics of gene transcription, ACS Synth. Biol. 2 (2013) 431–441, http://dx.doi.org/10.1021/sb300098w.
[168] D. Mishra, P.M. Rivera, A. Lin, D. Del Vecchio, R. Weiss, A load driver device for engineering modularity in biological networks, Nat. Biotechnol. (2014), http://dx.doi.org/10.1038/nbt.3044.
[169] D. Imburgio, M. Rong, K. Ma, W.T. McAllister, Studies of promoter recognition and start site selection by T7 RNA polymerase using a comprehensive collection of promoter variants, Biochemistry 39 (2000) 10419–10430, http://dx.doi.org/10.1021/bi000365w.
[170] G. Kudla, A.W. Murray, D. Tollervey, J.B. Plotkin, Coding-sequence determinants of gene expression in *Escherichia coli*, Science (New York, N.Y.) 324 (2009) 255–258, http://dx.doi.org/10.1126/science.1170160.
[171] W. Jeong, C. Kang, Start site selection at *lacUV5* promoter affected by the sequence context around the initiation sites, Nucleic Acids Res. 22 (1994) 4667–4672.
[172] D.B. Goodman, G.M. Church, S. Kosuri, Causes and effects of N-terminal codon bias in bacterial genes, Science 342 (2013) 475–479, http://dx.doi.org/10.1126/science.1241934.
[173] Y.-J. Chen, P. Liu, A.A.K. Nielsen, J.A.N. Brophy, K. Clancy, T. Peterson, et al., Characterization of 582 natural and synthetic terminators and quantification of their design constraints, Nat. Methods 10 (2013) 659–664, http://dx.doi.org/10.1038/nmeth.2515.
[174] G. Cambray, J.C. Guimaraes, V.K. Mutalik, C. Lam, Q.A. Mai, T. Thimmaiah, et al., Measurement and modeling of intrinsic transcription terminators, Nucleic Acids Res. 41 (2013) 5139–5148, http://dx.doi.org/10.1093/nar/gkt163.
[175] L. Pantoja-Hernández, J.C. Martínez-García, Retroactivity in the context of modularly structured biomolecular systems, Front. Bioeng. Biotechnol. (2015), http://dx.doi.org/10.3389/fbioe.2015.00085.
[176] S. Cardinale, M.P. Joachimiak, A.P. Arkin, Effects of genetic variation on the *E. coli* host-circuit interface, Cell Rep. 4 (2013) 231–237, http://dx.doi.org/10.1016/j.celrep.2013.06.023.
[177] L.B.A. Woodruff, N.R. Boyle, R.T. Gill, Engineering improved ethanol production in *Escherichia coli* with a genome-wide approach, Metab. Eng. 17 (2013) 1–11, http://dx.doi.org/10.1016/j.ymben.2013.01.006.
[178] W.H. Mather, J. Hasty, L.S. Tsimring, R.J. Williams, Translational cross talk in gene networks, Biophys. J. 104 (2013) 2564–2572, http://dx.doi.org/10.1016/j.bpj.2013.04.049.
[179] H. Dong, L. Nilsson, C.G. Kurland, Gratuitous overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction, J. Bacteriol. 177 (1995) 1497–1504.
[180] C. Gustafsson, S. Govindarajan, J. Minshull, Codon bias and heterologous protein expression, Trends Biotechnol. 22 (2004) 346–353, http://dx.doi.org/10.1016/j.tibtech.2004.04.006.
[181] A.M. Lanza, K.A. Curran, L.G. Rey, H.S. Alper, A condition-specific codon optimization approach for improved heterologous gene expression in *Saccharomyces cerevisiae*, BMC Syst. Biol. 8 (2014) 33, http://dx.doi.org/10.1186/1752-0509-8-33.
[182] H. Kobayashi, Inducible suppression of global translation by overuse of rare codons, Appl. Environ. Microbiol. (2015), AEM.03708–14, http://dx.doi.org/10.1128/AEM.03708-14.
[183] J.W. Chin, Expanding and reprogramming the genetic code of cells and animals, Annu. Rev. Biochem. (2014), http://dx.doi.org/10.1146/annurev-biochem-060713-035737.
[184] H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J.W. Chin, Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome, Nature 464 (2010) 441–444, http://dx.doi.org/10.1038/nature08817.
[185] R. Sorek, Y. Zhu, C.J. Creevey, M.P. Francino, P. Bork, E.M. Rubin, Genome-wide experimental determination of barriers to horizontal gene transfer, Science (New York, N.Y.) 318 (2007) 1449–1452, http://dx.doi.org/10.1126/science.1147112.
[186] A. Rizk, G. Batt, F. Fages, S. Soliman, A general computational method for robustness analysis with applications to synthetic gene networks, Bioinformatics 25 (2009) i169–i178, http://dx.doi.org/10.1093/bioinformatics/btp200.
[187] E. Andrianantoandro, S. Basu, D.K. Karig, R. Weiss, Synthetic biology: New engineering rules for an emerging discipline, Mol. Syst. Biol. 2 (2006) 1–14, http://dx.doi.org/10.1038/msb4100073.
[188] B.A. Renda, M.J. Hammerling, J.E. Barrick, Engineering reduced evolutionary potential for synthetic biology, Mol. Biosyst. 10 (2014) 1668–1678, http://dx.doi.org/10.1039/c3mb70606k.
[189] M. Kushwaha, H.M. Salis, A portable expression resource for engineering cross-species genetic circuits and pathways, Nat. Commun. 6 (2015) 7832, http://dx.doi.org/10.1038/ncomms8832.
[190] F. Ceroni, R. Algar, G.-B. Stan, T. Ellis, Quantifying cellular capacity identifies gene expression designs with reduced burden, Nat. Methods (2015), http://dx.doi.org/10.1038/nmeth.3339.
[191] R. Sarpeshkar, Analog synthetic biology, Philos. Transact. A Math. Phys. Eng. Sci. 372 (2014) 20130110, http://dx.doi.org/10.1098/rsta.2013.0110.
[192] G. Pósfai, G. Plunkett, T. Fehér, D. Frisch, G.M. Keil, K. Umenhoffer, et al., Emergent properties of reduced-genome *Escherichia coli*, Science (New York, N.Y.) 312 (2006) 1044–1046, http://dx.doi.org/10.1126/science.1126439.
[193] E.J. Olson, J.J. Tabor, Optogenetic characterization methods overcome key challenges in synthetic and systems biology, Nat. Chem. Biol. 10 (2014) 502–511, http://dx.doi.org/10.1038/nchembio.1559.
[194] D. Endy, Foundations for engineering biology, Nature 438 (2005) 449–453, http://dx.doi.org/10.1038/nature04342.
[195] A.P. Arkin, Setting the standard in synthetic biology, Nat. Biotechnol. 26 (2008) 771–774, http://dx.doi.org/10.1038/nbt0708-771.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[196] E.J. Olson, L.A. Hartsough, B.P. Landry, R. Shroff, J.J. Tabor, Characterizing bacterial gene circuit dynamics with optically programmed gene expression signals, Nat. Methods 11 (2014) 449–455, http://dx.doi.org/10.1038/nmeth.2884.
[197] J.-D. Pédelacq, S. Cabantous, T. Tran, T.C. Terwilliger, G.S. Waldo, Engineering and characterization of a superfolder green fluorescent protein, Nat. Biotechnol. 24 (2006) 79–88, http://dx.doi.org/10.1038/nbt1172.
[198] J.E. Toettcher, O.D. Weiner, W.A. Lim, Using optogenetics to interrogate the dynamic control of signal transmission by the Ras/Erk module, Cell 155 (2013) 1422–1434, http://dx.doi.org/10.1016/j.cell.2013.11.004.
[199] J.S. Paige, K.Y. Wu, S.R. Jaffrey, RNA mimics of green fluorescent protein, Science (New York, N.Y.) 333 (2011) 642–646, http://dx.doi.org/10.1126/science.1207339.
[200] W. Song, R.L. Strack, N. Svensen, S.R. Jaffrey, Plug-and-play fluorophores extend the spectral properties of spinach, J. Am. Chem. Soc. 136 (2014) 1198–1201, http://dx.doi.org/10.1021/ja410819x.
[201] R.L. Strack, W. Song, S.R. Jaffrey, Using Spinach-based sensors for fluorescence imaging of intracellular metabolites and proteins in living bacteria, Nat. Protoc. 9 (2014) 146–155, http://dx.doi.org/10.1038/nprot.2014.001.
[202] F. Akter, Y. Yokobayashi, RNA signal amplifier circuit with integrated fluorescence output, ACS Synth. Biol. (2014), http://dx.doi.org/10.1021/sb500314r.
[203] S. Bhadra, A.D. Ellington, A Spinach molecular beacon triggered by strand displacement, RNA 20 (2014) 1–12, http://dx.doi.org/10.1261/rna.045047.114.RNA.
[204] G.J. Nistala, K. Wu, C.V. Rao, K.D. Bhalerao, A modular positive feedback-based gene amplifier, J. Biol. Eng. 4 (2010) 4, http://dx.doi.org/10.1186/1754-1611-4-4.
[205] B. Wang, M. Barahona, M. Buck, Amplification of small molecule-inducible gene expression via tuning of intracellular receptor densities, Nucleic Acids Res. 43 (2015) 1955–1964, http://dx.doi.org/10.1093/nar/gku1388.
[206] E.A. Davidson, T. van Blarcom, M. Levy, A.D. Ellington, Emulsion based selection of T7 promoters of varying activity, Pac. Symp. Biocomput. 15 (2010) 433–443, http://dx.doi.org/10.1142/9789814295291_0045.
[207] S. Paul, A. Stang, K. Lennartz, M. Tenbusch, K. Überla, Selection of a T7 promoter mutant with enhanced *in vitro* activity by a novel multi-copy bead display approach for *in vitro* evolution, Nucleic Acids Res. 41 (2013) 1–11, http://dx.doi.org/10.1093/nar/gks940.
[208] J.T. Kittleson, S. Cheung, J.C. Anderson, Rapid optimization of gene dosage in *E. coli* using DIAL strains, J. Biol. Eng. 5 (2011) 10, http://dx.doi.org/10.1186/1754-1611-5-10.
[209] M.S. Lu, J.F. Mauser, K.E. Prehoda, Ultrasensitive synthetic protein regulatory networks using mixed decoys, ACS Synth. Biol. 1 (2012) 65–72, http://dx.doi.org/10.1021/sb200010w.
[210] J. Hasty, M. Dolnik, V. Rottschäfer, J.J. Collins, Synthetic gene network for entraining and amplifying cellular oscillations, Phys. Rev. Lett. 88 (2002) 148101, http://dx.doi.org/10.1103/PhysRevLett.88.148101.
[211] P.A. Silver, J.C. Way, F.H. Arnold, J.T. Meyerowitz, Synthetic biology: Engineering explored, Nature 509 (2014) 166–167, http://dx.doi.org/10.1038/509166a.
[212] E.L. Haseltine, F.H. Arnold, Synthetic gene circuits: Design with directed evolution, Annu. Rev. Biophys. Biomol. Struct. 36 (2007) 1–19, http://dx.doi.org/10.1146/annurev.biophys.36.040306.132600.
[213] M. Beguerisse-Diaz, B. Wang, R. Desikan, M. Barahona, Squeeze-and-breathe evolutionary Monte Carlo optimization with local search acceleration and its application to parameter fitting, J. R. Soc. Interface 9 (2012) 1925–1933, http://dx.doi.org/10.1098/rsif.2011.0767.
[214] A. Wong, H. Wang, C.L. Poh, R.I. Kitney, Layering genetic circuits to build a single cell, bacterial half adder, BMC Biol. 13 (2015) 40, http://dx.doi.org/10.1186/s12915-015-0146-0.
[215] T. Ellis, T. Adie, G.S. Baldwin, DNA assembly for synthetic biology: From parts to pathways and beyond, Integr Biol Quant Biosci from Nano to Macro, 32011 109–118, http://dx.doi.org/10.1039/c0ib00070a.
[216] T. Knight, Idempotent vector design for standard assembly of BioBricks doi: http://hdl.handle.net/1721.1/21168 2003.
[217] R.P. Shetty, D. Endy, T.F. Knight, Engineering BioBrick vectors from BioBrick parts, J. Biol. Eng. 2 (2008) 5, http://dx.doi.org/10.1186/1754-1611-2-5.
[218] J.C. Anderson, J.E. Dueber, M. Leguia, G.C. Wu, J.A. Goler, A.P. Arkin, et al., BglBricks: A flexible standard for biological part assembly, J. Biol. Eng. 4 (2010) 1, http://dx.doi.org/10.1186/1754-1611-4-1.
[219] T. Lee, R.A. Krupa, F. Zhang, M. Hajimorad, W.J. Holtz, N. Prasad, et al., BglBrick vectors and datasheets: A synthetic biology platform for gene expression, J. Biol. Eng. 5 (2011) 12, http://dx.doi.org/10.1186/1754-1611-5-12.
[220] M. Storch, A. Casini, B. Mackrow, H. Trewhitt, T. Ellis, G.S. Baldwin, BASIC: A new Biopart Assembly Standard for Idempotent Cloning provides accurate, single-tier DNA assembly for synthetic biology, ACS Synth. Biol. (2015), http://dx.doi.org/10.1021/sb500356d.
[221] J.P. Torella, F. Lienert, C.R. Boehm, J.-H. Chen, J.C. Way, P.A. Silver, Unique nucleotide sequence-guided assembly of repetitive DNA parts for synthetic biology applications, Nat. Protoc. 9 (2014) 2075–2089, http://dx.doi.org/10.1038/nprot.2014.145.
[222] M.Z. Li, S.J. Elledge, Harnessing homologous recombination *in vitro* to generate recombinant DNA via SLIC, Nat. Methods 4 (2007) 251–256, http://dx.doi.org/10.1038/nmeth1010.
[223] D.G. Gibson, L. Young, R.-Y. Chuang, J.C. Venter, C.A. Hutchison, H.O. Smith, Enzymatic assembly of DNA molecules up to several hundred kilobases, Nat. Methods 6 (2009) 343–345, http://dx.doi.org/10.1038/nmeth.1318.
[224] J. Quan, J. Tian, Circular polymerase extension cloning of complex gene libraries and pathways, PLoS One 4 (2009) e6441, http://dx.doi.org/10.1371/journal.pone.0006441.
[225] Y. Zhang, U. Werling, W. Edelmann, SLiCE: A novel bacterial cell extract-based DNA cloning method, Nucleic Acids Res. 40 (2012) 1–10, http://dx.doi.org/10.1093/nar/gkr1288.
[226] K. Mirzadeh, V. Martinez, S. Toddo, S. Guntur, M. Herrgard, A. Elofsson, et al., Enhanced protein production in *Escherichia coli* by optimization of cloning scars at the vector:coding sequence junction, ACS Synth. Biol. (2015), http://dx.doi.org/10.1021/acssynbio.5b00033.
[227] H.H. Wang, F.J. Isaacs, P.A. Carr, Z.Z. Sun, G. Xu, C.R. Forest, et al., Programming cells by multiplex genome engineering and accelerated evolution, Nature 460 (2009) 894–898, http://dx.doi.org/10.1038/nature08187.
[228] J.T. Stevens, J.M. Carothers, Designing RNA-based genetic control systems for efficient production from engineered metabolic pathways, ACS Synth. Biol. 4 (2015) 107–115, http://dx.doi.org/10.1021/sb400201u.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004

[229] J. Chappell, K.E. Watters, M.K. Takahashi, J.B. Lucks, A renaissance in RNA synthetic biology: New mechanisms, applications and tools for the future, Curr. Opin. Chem. Biol. 28 (2015) 47–56, http://dx.doi.org/10.1016/j.cbpa.2015.05.018.

[230] R.M. Davis, R.Y. Muller, K.A. Haynes, Can the natural diversity of quorum-sensing advance synthetic biology? Front. Bioeng. Biotechnol. 3 (2015) 1–10, http://dx.doi.org/10.3389/fbioe.2015.00030.

[231] K. De Roy, M. Marzorati, P. Van den Abbeele, T. Van de Wiele, N. Boon, Synthetic microbial ecosystems: An exciting tool to understand and apply microbial communities, Environ. Microbiol. 16 (2014) 1472–1481, http://dx.doi.org/10.1111/1462-2920.12343.

[232] A. Prindle, P. Samayoa, I. Razinkov, T. Danino, L.S. Tsimring, J. Hasty, A sensing array of radically coupled genetic "biopixels", Nature 481 (2012) 39–44, http://dx.doi.org/10.1038/nature10722.

[233] G.A. Khoury, J. Smadbeck, C.A. Kieslich, C.A. Floudas, Protein folding and *de novo* protein design for biotechnological applications, Trends Biotechnol. 32 (2014) 99–109, http://dx.doi.org/10.1016/j.tibtech.2013.10.008.

[234] S. Mirschel, K. Steinmetz, M. Rempel, M. Ginkel, E.D. Gilles, PROMOT: Modular modeling for systems biology, Bioinformatics 25 (2009) 687–689, http://dx.doi.org/10.1186/1471-2105-7-56.Klamt.

[235] A.D. Hill, J.R. Tomshine, E.M.B. Weeding, V. Sotiropoulos, Y.N. Kaznessis, SynBioSS: The synthetic biology modeling suite, Bioinformatics 24 (2008) 2551–2553, http://dx.doi.org/10.1093/bioinformatics/btn468.

[236] B.A. Funahashi, Y. Matsuoka, A. Jouraku, M. Morohashi, N. Kikuchi, H. Kitano, A versatile modeling tool for biochemical networks, Proc. IEEE 96 (2008) 1254–1265, http://dx.doi.org/10.1109/JPROC.2008.925458.

[237] R.W. Bradley, B. Wang, Designer cell signal processing circuits for biotechnology, New Biotechnol. 32 (2015) 635–643, http://dx.doi.org/10.1016/j.nbt.2014.12.009.

[238] R. Daniel, J.R. Rubens, R. Sarpeshkar, T.K. Lu, Synthetic analog computation in living cells, Nature 497 (2013) 619–623, http://dx.doi.org/10.1038/nature12148.

[239] L. Zhu, Y. Zhu, Y. Zhang, Y. Li, Engineering the robustness of industrial microbes through synthetic biology, Trends Microbiol. 20 (2012) 94–101, http://dx.doi.org/10.1016/j.tim.2011.12.003.

Please cite this article as: R. W. Bradley, et al., Tools and Principles for Microbial Gene Circuit Engineering, J. Mol. Biol. (2015), http://dx.doi.org/10.1016/j.jmb.2015.10.004